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(54) Title: STEREOISOMERS OF CpG OLIGONUCLEOTIDES AND RELATED METHODS (57) Abstract <p>The interactions of nucleic acids with proteins can be selective for the R stereoisomer, the S stereoisomer, or can be stereoindependent. The present invention demonstrates that the S stereoisomer of CpG containing DNA is active in mediating the immune stimulatory effects of CpG DNA. The invention provides methods of use of a pure stereoisomer or of DNA enriched for this form for clinical applications for CpG DNA, such as vaccine adjuvants, immune activators for the prevention or treatment of retroviral, viral, parasitic or fungal diseases, or cancer immunotherapy, immunotherapy of allergic and asthmatic diseases, etc. The invention also provides methods of use for R stereoisomer DNA to oppose the immune stimulatory effects of CpG DNA. Such R stereoisomers are useful in the treatment of diseases such as Sepsis syndrome, intestinal inflammatory diseases, psoriasis, gingivitis, systemic lupus erythematosus and other autoimmune diseases.</p>		

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STEREOMERS OF CpG OLIGONUCLEOTIDES AND RELATED METHODS

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Field of the Invention

The present invention relates generally to stereoisomers, and in particular the S isoform of CpG oligonucleotides. The invention also relates to methods of using the stereoisomers of CpG oligonucleotides.

10

Background of the Invention

Aside from its role in carrying the genetic code, DNA has recently been shown to function as a signaling molecule (Krieg, A.M., 1998, *Biodrugs*). The immune systems of higher eukaryotes appear to have evolved a mechanism to detect prokaryotic nucleic acids based on their content of unmethylated CpG dinucleotides in particular base contexts (Krieg, A.K., et al., 1995, *Nature*, 374:546). Unmethylated CpG dinucleotides are common in bacterial DNA, but are underrepresented ("CpG suppression") and are methylated in vertebrate DNA (Bird, A.P., 1987, *Trends in Genetics*, 3:342). DNA containing these unmethylated CpG dinucleotides in immune stimulatory base contexts ("CpG motifs") triggers humoral immunity by inducing B cell activation, resistance to activation-induced apoptosis, and IL-6 and IgM secretion (Krieg, A.K., et al., 1995, *Nature*, 374:546; Yi, A.K., et al., 1996, *J. Immunol.*, 157:5394; and Klinman, D., et al., 1996, *Proc. Natl. Acad. Sci. USA*, 93:2879). Such CpG DNA also directly activates monocytes and macrophage to secrete Th1-like cytokines (Ballas, Z.K., et al., 1996, *J. Immunol.*, 157:1840; Cowdery, J.S., et al., 1996, *J. Immunol.*, 156:4570; and Halpern, M.D., et al., 1996, *Cell. Immunol.*, 167:72). This leads to the activation of natural killer (NK) cell lytic activity and interferon-gamma (IFN-gamma) secretion (Ballas, Z.K., et al., 1996, *J. Immunol.*, 157:1840; Cowdery, J.S., et al., 1996, *J. Immunol.*, 156:4570; and Chace, J.H., 1997, *Clin. Immunol. Immunopath.*, 84:185). The effects of CpG oligonucleotides on immune activation have been described in commonly assigned and having common inventorship, U.S. Patent Applications U.S. Serial No. 08/960,774, filed October 30, 1997, which is a continuation-in-part of U.S. Serial No. 08/738,652, filed October 30, 1996, which is a continuation-in-part of U.S. Serial No.

08/386,063, filed February 7, 1995, and which is a continuation-in-part of U.S. Serial No. 08/276,358, filed July 15, 1994, each of which is hereby incorporated by reference in its entirety.

Native DNA has a phosphodiester backbone and is rapidly degraded by a large number of exo- and endonucleases in living cells. CpG oligonucleotides having a nuclease-resistant phosphorothioate backbone, however, have markedly enhanced immune stimulatory effects (Krieg, A.M., et al., 1996, *Antisense Res. Dev.*, 6:133 and Zhao, Q., et al., 1996, *Biochem. Pharmac.*, 51:173). Although the backbone of normal phosphodiester DNA has no chiral centers, the phosphorothioate modification replaces one of the two non-bridging oxygens on the phosphodiester bond with a sulphur atom, which creates a new chiral center. Each internucleotide linkage in a phosphorothioate DNA backbone can be either an R or an S stereoisomer.

Since native DNA is achiral, it was not known whether proteins that interact with DNA would do so in a stereoselective or stereoindependent fashion. Several studies have been performed using chirally pure phosphorothioate oligonucleotides to investigate this question. Some interactions with DNA were found to be specific for the R stereoisomer. For example, the degradation of DNA by snake venom phosphodiesterase or 3' exonuclease is selective for the R stereoisomer and is extremely inefficient with the S stereoisomer (Bergers, P.M., and F. Eckstein, 1978, *Proc. Natl. Acad. Sci. USA*, 75:4798; Bergers, P.M., et al., 1979, *Eur. J. Biochem.*, 100:585; Tang, J.A., et al., 1995, *Nucleosides and Nucleotides*, 14:985; Koziolkiewicz, M., et al., 1997, *Antisense and Nucleic Acid Drug Devel.*, 7:43; and Gilar, M.A., et al., 1998, *Antisense and Nucleic Acid Drug Devel.*, 8:35). Likewise, the RNase H cleavage of RNA/DNA duplexes is selective for the R stereoisomer of the DNA component (Koziolkiewicz, M., 1995, *Nucleic Acids Res.*, 23:5000). The hybridization of oligonucleotides to complementary RNA has been reported to be higher for the R stereoisomer than for the S (Tang, J., et al., 1995, *Nucleosides and Nucleotides*, 14:985 and Koziolkiewicz, M., et al., 1995, *Nucleic Acids Res.*, 23:5000) as has the antisense activity of the oligonucleotides in some experimental systems (Fearon, K.L., et al., 1997, *Oligonucleotides as Therapeutic Agents*, Wiley, Chichester (Ciba Foundation Symposium 209), New York, p. 19).

On the other hand, some experimental systems recognize only the S stereoisomer. For example, DNA-dependent RNA polymerase preferentially binds the S stereoisomer (Bergers, P.M., and F. Eckstein, 1978, *Proc. Natl. Acad. Sci. USA*, 75:4798). The nuclease P1 and Eco R1 endonuclease both preferentially bind and cleave the S stereoisomer (Griffith, S.A.D., 1987,

Nucleic Acids Res., 15:4145; Lesser, D.R., et al., 1992, *J. Biol. Chem.*, 267:24810; and Kurpiewski, M.R., et al., 1996, *Biochem.*, 35:8846). Of note, the T7 RNA polymerase also uses S bases and cannot use R, despite the fact that the RNA produced by this enzyme is in the R stereoisomer (Griffith, A.D., et al., 1987, *Nucleic Acids Res.*, 15:4145). Certain four-stranded
5 structures of DNA are reportedly more stable with the S stereoisomer (Kanehara, H., et al., 1997, *Biochem.*, 36:1790) and some S stereoisomers have also been reported to have greater antisense activity in cells and *in vivo* in certain experimental systems (Stec, W.J., 1997, *Antisense and Nucleic Acid Drug Devel.*, 7:567).

In contrast to these stereoselective biological activities, the interaction of nucleic acids
10 with creatine kinase can be selective for either the R or the S stereoisomer depending on the cation present (Bergers, P.M., and F. Eckstein, 1980). On the other hand, the binding of phosphorothioate oligonucleotides to basic fibroblast growth factor, CD4, laminin, and fibronectin is not stereoselective (Benimetskaya, L., et al., 1995, *Nucleic Acids Res.*, 23:4239). Thus, the interactions of nucleic acids with proteins can be selective for the R stereoisomer, the S
15 stereoisomer, or can be stereoindependent.

Summary of the Invention

The present invention relates to stereoisomers of CpG oligonucleotides and methods of use. The invention is based in part on the discovery that only the S stereoisomer of phosphate
20 backbone modified CpG containing oligonucleotide is active in mediating the immune response. It was discovered that both a stereorandom and a chirally pure R stereoisomer of a CpG containing nucleic acid had reduced immune stimulating properties compared to a chirally pure S stereoisomer of the identical sequence. Addition of the R stereoisomer to cells that were being stimulated with the S stereoisomer caused a dose-dependent reduction in the immune stimulating
25 effect. Thus, it is possible according to the methods of the invention to administer lower doses of CpG compositions which are chirally pure forms of the S stereoisomer than it was to administer CpG compositions which include mixtures of the S and R stereoisomers or of the R stereoisomer alone. These findings have important implications for the clinical development of immune stimulatory CpG containing nucleic acids.

30 In one aspect the invention is a composition of an immunostimulatory nucleic acid having a sequence including at least the following formula:



wherein N is a nucleic acid sequence composed of from about 0-25 nucleotides.

In some aspects or embodiments the immunostimulatory nucleic acid is double stranded and in others it is single stranded.

The composition includes immunostimulatory nucleic acids having identical sequences in some embodiments. In other embodiments the composition includes immunostimulatory nucleic acids having at least two different sequences.

The invention encompasses in other aspects methods of using the immunostimulatory oligonucleotides. These methods include methods of inducing an antigen-specific immune response in a subject, methods for redirecting a subject's immune response from a Th2 to a Th1, methods for treating asthma in a subject, methods for desensitizing a subject against the occurrence of an allergic reaction in response to contact with an allergen, methods for activating an immune cell, methods for treating a cancer, methods for enhancing recovery of bone marrow in a cancer therapy subject, methods for stimulating an immune response in a subject having a

cancer, the method of the type involving antigen dependent cellular cytotoxicity (ADCC), the improvement, methods for inducing cytokine production in a subject, methods of stimulating natural killer cell lytic activity, methods of inducing a Th1-type immune response in a subject, and methods for inducing a mucosal immune response.

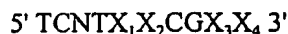
5 In another embodiment the nucleic acid has a sequence including at least the formula
GTCGTX₄.

In other aspects the invention is based on the finding that the R stereoisomer of CpG containing nucleic acids reduces the ability of the S stereoisomer to induce an immune response. A CpG nucleic acid composed partially or completely of the R stereoisomer may be used to
10 inhibit an immune response when it is desirable to do so. For instance, when CpG nucleic acid has been administered in too high of a dose and causes more of an immune response than is desirable the R stereoisomer may be administered to antagonize the immune stimulating effect of CpG. Alternatively if only a transient immune stimulating effect is desired the R stereoisomer can be administered to reduce the immune stimulation. Thus it is possible to regulate the timing
15 of a CpG induced immune response by administering R stereoisomer at the appropriate time. The R stereoisomer of CpG is also useful for the therapeutic treatment of disorders mediated by an excessive immune response. Thus the R stereoisomer can be used as a therapeutic to treat inflammatory disorders.

In another aspect the invention is a composition of an immunoinhibitory nucleic acid
20 having a sequence including at least the following formula:



wherein C and G are unmethylated, wherein X₁, X₂, X₃ and X₄ are nucleotides, wherein at least two nucleotides have a phosphate backbone modification forming a chiral center and wherein a plurality of the chiral centers have R chirality. In some embodiments the immunoinhibitory
25 nucleic acid has a sequence including at least the following formula:



wherein N is a nucleic acid sequence composed of from about 0-25 nucleotides.

In some embodiments X₁X₂ are nucleotides selected from the group consisting of: TpA, ApA, ApC, ApG, and GpG. In other embodiments X₃X₄ are nucleotides selected from the group
30 consisting of: TpT, TpA, TpG, ApA, ApG, GpA, and CpA. In yet other embodiments X₁X₂ are nucleotides selected from the group consisting of: TpT, TpG, ApT, GpC, CpC, CpT, TpC, GpT

and CpG; X_3 is a nucleotide selected from the group consisting of A and T and X_4 is a nucleotide, but wherein when X_1X_2 is TpC, GpT, or CpG, X_3X_4 is not TpC, ApT or ApC.

In some aspects or embodiments the immunoinhibitory nucleic acid is double stranded and in others it is single stranded.

5 The composition in some embodiments has less than all of the nucleotides with a backbone modification. In other embodiments less than all of the chiral centers have R chirality. In preferred embodiments at least 50%, 75% or 90% of the nucleotides have backbone modifications. In other preferred embodiments at least 60%, 75% or 90% of the chiral centers have R chirality.

10 The composition includes immunoinhibitory nucleic acids having identical sequences in some embodiments. In other embodiments the composition includes immunoinhibitory nucleic acids having at least two different sequences.

 The composition may also include other compounds, such as an antigen, a cytokine such as GM-CSF, IL-4, IL-18, IFN α , TNF α , Flt3 ligand, and IL-3, an antiviral, an antibacterial, or a
15 non-nucleic acid adjuvant.

 The invention also encompasses methods of preventing an immune response in a subject and treating inflammatory diseases using the immunoinhibitory oligonucleotides of the invention.

 According to yet another aspect the invention is a method for inducing antigen non-
20 specific innate immune activation and broad spectrum resistance to infectious challenge.

 Each of the limitations of the invention can encompass various embodiments of the invention. It is, therefore, anticipated that each of the limitations of the invention involving any one element or combinations of elements can be included in each aspect of the invention.

25 Detailed Description of the Invention

 The invention in one aspect involves the finding that the S stereoisomer of CpG containing nucleic acids is more effective in mediating immune stimulatory effects than the R stereoisomer or mixtures thereof. These findings enable, among other things, the use of lower doses of CpG preparations that are partially, predominantly, or completely composed of the S
30 stereoisomer.

CpG containing nucleic acids have been found to be novel therapeutic and prophylactic compositions that stimulate the immune system to treat cancer, infectious diseases, allergy, asthma and other disorders and to help protect against opportunistic infections following cancer chemotherapies. The strong yet balanced, cellular and humoral immune responses that result from CpG stimulation reflect the body's own natural defense system against invading pathogens and cancerous cells. CpG sequences, while relatively rare in human DNA are commonly found in the DNA of infectious organisms such as bacteria. The human immune system has apparently evolved to recognize CpG sequences as an early warning sign of infection, and to initiate an immediate and powerful immune response against invading pathogens without causing adverse reactions frequently seen with other immune stimulatory agents. Thus CpG containing nucleic acids, relying on this innate immune defense mechanism can utilize a unique and natural pathway for immune therapy. The effects of CpG nucleic acids on immune modulation were discovered by the inventor of the instant patent application and have been described extensively in co-pending patent applications, such as U.S. Patent Application Serial Nos:

08/386,063 filed on 02/07/95 (and related PCT US95/01570); 08/738,652 filed on 10/30/96; 08/960,774 filed on 10/30/97 (and related PCT/US97/19791, WO 98/18810); 09/191,170 filed on 11/13/98; 09/030,701 filed on 02/25/98 (and related PCT/US98/03678; 09/082,649 filed on 05/20/98 (and related PCT/US98/10408); 09/325,193 filed on 06/03/99 (and related PCT/US98/04703); 09/286,098 filed on 04/02/99 (and related PCT/US99/07335); 09/306,281 filed on 05/06/99 (and related PCT/US99/09863). The entire contents of each of these patents and patent applications is hereby incorporated by reference.

The findings of the instant invention are applicable to all of the above described uses of CpG containing nucleic acids as well as any other known use for CpG nucleic acids. The invention involves the discovery that CpG oligonucleotides with a modified phosphate backbone having S chirality have superior immune activating properties to mixtures of R and S chiral oligonucleotides or oligonucleotides having R chirality. Thus the invention is useful for any method for stimulating the immune system using CpG oligonucleotides having S chirality.

A CpG oligonucleotide is an oligonucleotide which includes at least one unmethylated CpG dinucleotide. An oligonucleotide containing at least one unmethylated CpG dinucleotide is a nucleic acid molecule which contains an unmethylated cytosine-guanine dinucleotide sequence (i.e. "CpG DNA" or DNA containing a 5' cytosine followed by 3' guanosine and linked by a

phosphate bond) and activates the immune system. The CpG oligonucleotides can be double-stranded or single-stranded. Generally, double-stranded molecules are more stable *in vivo*, while single-stranded molecules have increased immune activity. Thus in some aspects of the invention it is preferred that the oligonucleotide be single stranded and in other aspects it is preferred that the oligonucleotide be double stranded. The terms CpG oligonucleotide or CpG nucleic acid as used herein refer to an immunostimulatory CpG oligonucleotide or a nucleic acid unless otherwise indicated.

The terms "nucleic acid" and "oligonucleotide" are used interchangeably to mean multiple nucleotides (i.e. molecules comprising a sugar (e.g. ribose or deoxyribose) linked to a phosphate group and to an exchangeable organic base, which is either a substituted pyrimidine (e.g. cytosine (C), thymine (T) or uracil (U)) or a substituted purine (e.g. adenine (A) or guanine (G)). As used herein, the terms refer to oligoribonucleotides as well as oligodeoxyribonucleotides. The terms shall also include polynucleosides (i.e. a polynucleotide minus the phosphate) and any other organic base containing polymer. Nucleic acid molecules can be obtained from existing nucleic acid sources (e.g. genomic or cDNA), but are preferably synthetic (e.g. produced by oligonucleotide synthesis). The entire CpG oligonucleotide can be unmethylated or portions may be unmethylated but at least the C of the 5' CG 3' must be unmethylated.

In one preferred embodiment the invention provides a CpG oligonucleotide represented by at least the formula:



wherein X_1 , X_2 , X_3 , and X_4 are nucleotides. In one embodiment X_2 is adenine, guanine, or thymine. In another embodiment X_3 is cytosine, adenine, or thymine.

In another embodiment the invention provides an isolated CpG oligonucleotide represented by at least the formula:



wherein X_1 , X_2 , X_3 , and X_4 are nucleotides and N_1 and N_2 are nucleic acid sequences composed of from about 0-25 N's each. In one embodiment X_1X_2 are nucleotides selected from the group consisting of: GpT, GpG, GpA, ApA, ApT, ApG, CpT, CpA, CpG, TpA, TpT, and TpG; and X_3X_4 are nucleotides selected from the group consisting of: TpT, CpT, ApT, TpG, ApG, CpG, TpC, ApC, CpC, TpA, ApA, and CpA. Preferably X_1X_2 are GpA or GpT and

X_3X_4 are TpT. In other embodiments X_1 or X_2 or both are purines and X_3 or X_4 or both are pyrimidines or X_1X_2 are GpA and X_3 or X_4 or both are pyrimidines. In another preferred embodiment X_1X_2 are nucleotides selected from the group consisting of: TpA, ApA, ApC, ApG, and GpG. In yet another embodiment X_3X_4 are nucleotides selected from the group consisting of: TpT, CpT, TpA, TpG, ApA, ApG, GpA, and CpA. X_1X_2 in another embodiment are nucleotides selected from the group consisting of: TpT, TpG, ApT, GpC, CpC, CpT, TpC, GpT and CpG; X_3 is a nucleotide selected from the group consisting of A and T and X_4 is a nucleotide, but wherein when X_1X_2 is TpC, GpT, or CpG, X_3X_4 is not TpC, ApT or ApC.

In another embodiment N_1 and N_2 of the nucleic acid do not contain a CCGG or CGCG quadmer or more than one CCG or CGG trimer. The effect of a CCGG or CGCG quadmer or more than one CCG or CGG trimer depends in part on the status of the oligonucleotide backbone. The synthetic oligonucleotides may not, in some embodiments, include a CCGG or CGCG quadmer or more than one CCG or CGG trimer at or near the 5' and/or 3' terminals. For instance, if the oligonucleotide has a phosphodiester backbone or a chimeric backbone the inclusion of these sequences in the oligonucleotide will only have minimal if any affect on the biological activity of the oligonucleotide. If the backbone is completely phosphorothioate (or other phosphate modification) or significantly phosphorothioate then the inclusion of these sequences may have more influence on the biological activity or the kinetics of the biological activity. In the case when the CpG oligonucleotide is administered in conjunction with an antigen which is encoded in a nucleic acid vector, it is preferred that the backbone of the CpG oligonucleotide be a chimeric combination of phosphodiester and phosphorothioate (or other phosphate modification). The cell may have a problem taking up a plasmid vector in the presence of completely phosphorothioate oligonucleotide. Thus when both a vector and an oligonucleotide are delivered to a subject, it is preferred that the oligonucleotide have a chimeric backbone or have a phosphorothioate backbone but that the plasmid associated with a vehicle that delivers it directly into the cell, thus avoiding the need for cellular uptake. Such vehicles are known in the art and include, for example, liposomes and gene guns.

In another preferred embodiment the CpG oligonucleotide has the sequence 5'TCN₁TX₁X₂CGX₃X₄3'. The CpG oligonucleotides of the invention in some embodiments include X_1X_2 selected from the group consisting of GpT, GpG, GpA and ApA and X_3X_4 is selected from the group consisting of TpT, CpT and TpC.

For facilitating uptake into cells, CpG containing oligonucleotides are preferably in the range of 8 to 100 bases in length. However, nucleic acids of any size greater than 8 nucleotides (even many kb long) are capable of inducing an immune response according to the invention if sufficient immunostimulatory motifs are present, since larger nucleic acids are degraded into
 5 oligonucleotides inside of cells. Preferably the CpG oligonucleotide is in the range of between 8 and 100 and in some embodiments between 8 and 30 nucleotides in size.

"Palindromic sequence" shall mean an inverted repeat (i.e. a sequence such as ABCDEE'D'C'B'A' in which A and A' are bases capable of forming the usual Watson-Crick base pairs. *In vivo*, such sequences may form double-stranded structures. In one embodiment
 10 the CpG oligonucleotide contains a palindromic sequence. A palindromic sequence used in this context refers to a palindrome in which the CpG is part of the palindrome, and preferably is the center of the palindrome. In another embodiment the CpG oligonucleotide is free of a palindrome. A CpG oligonucleotide that is free of a palindrome is one in which the CpG dinucleotide is not part of a palindrome. Such an oligonucleotide may include a palindrome in
 15 which the CpG is not the center of the palindrome.

The CpG nucleic acid sequences of the invention are those broadly described above as well as disclosed in PCT Published Patent Applications PCT/US95/01570 and PCT/US97/19791 claiming priority to U.S. Serial Nos. 08/386,063 and 08/960,774, filed on February 7, 1995 and October 30, 1997 respectively. Exemplary sequences include but are not limited to those
 20 immunostimulatory sequences shown in Table 1.

Table 1

	GCTAGACGTTAGCGT;	(SEQ ID NO: 1)
	GCTAGATGTTAGCGT;	(SEQ ID NO: 2)
	GCTAGACGTTAGCGT;	(SEQ ID NO: 3)
25	GCTAGACGTTAGCGT;	(SEQ ID NO: 4)
	GCATGACGTTGAGCT;	(SEQ ID NO: 5)
	ATGGAAGGTCCAGCGTTCTC;	(SEQ ID NO: 6)
	ATCGACTCTCGAGCGTTCTC;	(SEQ ID NO: 7)
	ATCGACTCTCGAGCGTTCTC;	(SEQ ID NO: 8)
30	ATCGACTCTCGAGCGTTCTC;	(SEQ ID NO: 9)
	ATGGAAGGTCCAACGTTCTC;	(SEQ ID NO: 10)
	GAGAACGCTGGACCTTCCAT;	(SEQ ID NO: 11)
	GAGAACGCTCGACCTTCCAT;	(SEQ ID NO: 12)
	GAGAACGCTCGACCTTCGAT;	(SEQ ID NO: 13)
35	GAGAACGCTGGACCTTCCAT;	(SEQ ID NO: 14)
	GAGAACGATGGACCTTCCAT;	(SEQ ID NO: 15)
	GAGAACGCTCCAGCACTGAT;	(SEQ ID NO: 16)
	TCCATGTCTGGTCTCTGATGCT;	(SEQ ID NO: 17)

	TCCATGTCGGTCCTGATGCT;	(SEQ ID NO: 18)
	TCCATGACGTTCCCTGATGCT;	(SEQ ID NO: 19)
	TCCATGTCGGTCCTGCTGAT;	(SEQ ID NO: 20)
	TCAACGTT;	(SEQ ID NO: 21)
5	TCAGCGCT;	(SEQ ID NO: 22)
	TCATCGAT;	(SEQ ID NO: 23)
	TCTTCGAA;	(SEQ ID NO: 24)
	CAACGTT;	(SEQ ID NO: 25)
	CCAACGTT;	(SEQ ID NO: 26)
10	AACGTTCT;	(SEQ ID NO: 27)
	TCAACGTC;	(SEQ ID NO: 28)
	ATGGACTCTCCAGCGTTCTC;	(SEQ ID NO: 29)
	ATGGAAGGTCCAACGTTCTC;	(SEQ ID NO: 30)
	ATCGACTCTCGAGCGTTCTC;	(SEQ ID NO: 31)
15	ATGGAGGCTCCATCGTTCTC;	(SEQ ID NO: 32)
	ATCGACTCTCGAGCGTTCTC;	(SEQ ID NO: 33)
	ATCGACTCTCGAGCGTTCTC;	(SEQ ID NO: 34)
	TCCATGTCGGTCCTGATGCT;	(SEQ ID NO: 35)
	TCCATGCCGGTCCTGATGCT;	(SEQ ID NO: 36)
20	TCCATGGCGGTCCTGATGCT;	(SEQ ID NO: 37)
	TCCATGACGGTCCTGATGCT;	(SEQ ID NO: 38)
	TCCATGTCGATCCTGATGCT;	(SEQ ID NO: 39)
	TCCATGTCGCTCCTGATGCT;	(SEQ ID NO: 40)
	TCCATGTCGTCCCTGATGCT;	(SEQ ID NO: 41)
25	TCCATGACGTGCCTGATGCT;	(SEQ ID NO: 42)
	TCCATAACGTTCCCTGATGCT;	(SEQ ID NO: 43)
	TCCATGACGTCCCTGATGCT;	(SEQ ID NO: 44)
	TCCATCACGTGCCTGATGCT;	(SEQ ID NO: 45)
	GGGGTCAACGTTGACGGGG;	(SEQ ID NO: 46)
30	GGGGTCAGTCGTGACGGGG;	(SEQ ID NO: 47)
	GCTAGACGTTAGTGT;	(SEQ ID NO: 48)
	TCCATGTCGTTCCCTGATGCT;	(SEQ ID NO: 49)
	ACCATGGACGATCTGTTTCCCCTC;	(SEQ ID NO: 50)
	TCTCCCAGCGTGCGCCAT;	(SEQ ID NO: 51)
35	ACCATGGACGAACTGTTTCCCCTC;	(SEQ ID NO: 52)
	ACCATGGACGAGCTGTTTCCCCTC;	(SEQ ID NO: 53)
	ACCATGGACGACCTGTTTCCCCTC;	(SEQ ID NO: 54)
	ACCATGGACGTA CTGTTTCCCCTC;	(SEQ ID NO: 55)
	ACCATGGACGGTCTGTTTCCCCTC;	(SEQ ID NO: 56)
40	ACCATGGACGTTCTGTTTCCCCTC;	(SEQ ID NO: 57)
	CACGTTGAGGGGCAT;	(SEQ ID NO: 58)
	TCAGCGTGCGCC;	(SEQ ID NO: 59)
	ATGACGTTCCCTGACGTT;	(SEQ ID NO: 60)
	TCTCCCAGCGGGCGCAT;	(SEQ ID NO: 61)
45	TCCATGTCGTTCCCTGTCGTT;	(SEQ ID NO: 62)
	TCCATAGCGTTCCTAGCGTT;	(SEQ ID NO: 63)
	TCGTCGCTGTCTCCCCTTCTT;	(SEQ ID NO: 64)
	TCCTGACGTTCCCTGACGTT;	(SEQ ID NO: 65)

	TCCTGTCGTTCCCTGTCGTT;	(SEQ ID NO: 66)
	TCCATGTCGTTTTTGTGCGTT;	(SEQ ID NO: 67)
	TCCTGTCGTTCCCTGTCGTT;	(SEQ ID NO: 68)
	TCCTTGTGCGTTCCCTGTCGTT;	(SEQ ID NO: 69)
5	TCCTGTCGTTTTTTGTGCGTT;	(SEQ ID NO: 70)
	TCGTGCGCTGTCTGCCCTTCTT;	(SEQ ID NO: 71)
	TCGTGCGCTGTTGTGCGTTTCTT;	(SEQ ID NO: 72)
	TCCATGCGTGCGTGCGTTTT;	(SEQ ID NO: 73)
	TCCATGCGTTGCGTTGCGTT;	(SEQ ID NO: 74)
10	TCCACGACGTTTTTCGACGTT;	(SEQ ID NO: 75)
	TCGTGCGTTGTGCGTTGTGCGTT;	(SEQ ID NO: 76)
	TCGTGCGTTTTGTGCGTTTTGTGCGTT;	(SEQ ID NO: 77)
	TCGTGCGTTGTGCGTTTTGTGCGTT;	(SEQ ID NO: 78)
	GCGTGCGTTGTGCGTTGTGCGTT;	(SEQ ID NO: 79)
15	TGTCGTTTGTGCGTTTGTGCGTT;	(SEQ ID NO: 80)
	TGTCGTTGTGCGTTGTGCGTTGTGCGTT;	(SEQ ID NO: 81)
	TGTCGTTGTGCGTTGTGCGTT;	(SEQ ID NO: 82)
	TCGTGCGTCGTCGTT;	(SEQ ID NO: 83)
	TGTCGTTGTGCGTT;	(SEQ ID NO: 84)
20	TCCATAGCGTTCCTAGCGTT;	(SEQ ID NO: 85)
	TCCATGACGTTCCCTGACGTT;	(SEQ ID NO: 86)
	GTCGYT;	(SEQ ID NO: 87)
	TGTCGYT;	(SEQ ID NO: 88)
	AGCTATGACGTTCCAAGG;	(SEQ ID NO: 89)
25	TCCATGACGTTCCCTGACGTT;	(SEQ ID NO: 90)
	ATCGACTCTCGAACGTTCTC;	(SEQ ID NO: 91)
	TCCATGTCGGTCCTGACGCA;	(SEQ ID NO: 92)
	TCTTCGAT;	(SEQ ID NO: 93)
	ATAGGAGGTCCAACGTTCTC;	(SEQ ID NO: 94)

30

For use in the instant invention, the nucleic acids (both the S and R chiral nucleic acids) can be synthesized *de novo* using any of a number of procedures well known in the art. For example, the b-cyanoethyl phosphoramidite method (Beaucage, S.L., and Caruthers, M.H., *Tet. Let.* 22:1859, 1981); nucleoside H-phosphonate method (Garegg *et al.*, *Tet. Let.* 27:4051-4054, 1986; Froehler *et al.*, *Nucl. Acid. Res.* 14:5399-5407, 1986, ; Garegg *et al.*, *Tet. Let.* 27:4055-4058, 1986, Gaffney *et al.*, *Tet. Let.* 29:2619-2622, 1988). These chemistries can be performed by a variety of automated oligonucleotide synthesizers available in the market. These oligonucleotides are referred to as synthetic oligonucleotides. Alternatively, CpG dinucleotides can be produced on a large scale in plasmids, (see Sambrook, T., *et al.*, "Molecular Cloning: A Laboratory Manual", Cold Spring Harbor laboratory Press, New York, 1989) and separated into smaller pieces or administered whole. Oligonucleotides can be prepared from existing nucleic acid sequences (*e.g.*, genomic or cDNA) using known techniques, such as those employing

40

restriction enzymes, exonucleases or endonucleases. Oligonucleotides prepared in this manner are referred to as isolated oligonucleotide. The term CpG oligonucleotide or CpG nucleic acid (both the S and R chiral nucleic acids) encompasses both synthetic and isolated CpG oligonucleotides.

- 5 For use *in vivo*, nucleic acids (both the S and R chiral nucleic acids) are preferably relatively resistant to degradation (*e.g.*, are stabilized). A "stabilized nucleic acid molecule" shall mean a nucleic acid molecule that is relatively resistant to *in vivo* degradation (*e.g.* via an exo- or endo-nuclease). Stabilization can be a function of length or secondary structure. Unmethylated CpG oligonucleotides that are tens to hundreds of kbs long are relatively resistant to *in vivo* degradation. For shorter CpG oligonucleotides, secondary structure can stabilize and increase their effect. For example, if the 3' end of an oligonucleotide has self-complementarity to an upstream region, so that it can fold back and form a sort of stem loop structure, then the oligonucleotide becomes stabilized and therefore exhibits more activity.

- Alternatively, nucleic acid stabilization can be accomplished via phosphate backbone
15 modifications. Preferred stabilized oligonucleotides of the instant invention have a modified backbone. It has been demonstrated that modification of the oligonucleotide backbone provides enhanced activity of the CpG oligonucleotides when administered *in vivo*. These stabilized structures are preferred because the chiral CpG molecules of the invention have at least a partial modified backbone. CpG constructs, including at least two phosphorothioate linkages at the 5'
20 end of the oligonucleotide and multiple phosphorothioate linkages at the 3' end, preferably 5, provide maximal activity and protect the oligonucleotide from degradation by intracellular exo- and endo-nucleases. Other modified oligonucleotides include phosphodiester modified oligonucleotides, combinations of phosphodiester and phosphorothioate oligonucleotide, methylphosphonate, methylphosphorothioate, phosphorodithioate, and combinations thereof.
25 Each of these combinations and their particular effects on immune cells is discussed in more detail in PCT Published Patent Applications PCT/US95/01570 and PCT/US97/19791 claiming priority to U.S. Serial Nos. 08/386,063 and 08/960,774, filed on February 7, 1995 and October 30, 1997 respectively, the entire contents of which is hereby incorporated by reference. It is believed that these modified oligonucleotides may show more stimulatory activity due to
30 enhanced nuclease resistance, increased cellular uptake, increased protein binding, and/or altered intracellular localization.

Modified backbones such as phosphorothioates may be synthesized using automated techniques employing either phosphoramidate or H-phosphonate chemistries. Aryl- and alkyl-phosphonates can be made, *e.g.*, as described in U.S. Patent No. 4,469,863; and alkylphosphotriesters (in which the charged oxygen moiety is alkylated as described in U.S. Patent No. 5,023,243 and European Patent No. 092,574) can be prepared by automated solid phase synthesis using commercially available reagents. Methods for making other DNA backbone modifications and substitutions have been described (Uhlmann, E. and Peyman, A., *Chem. Rev.* 20:544, 1990; Goodchild, J., *Bioconjugate Chem.* 1:165, 1990).

Both phosphorothioate and phosphodiester oligonucleotides containing CpG motifs are active in immune cells. However, based on the concentration needed to induce CpG specific effects, the nuclease resistant phosphorothioate backbone CpG oligonucleotides are more potent (2 $\mu\text{g/ml}$ for the phosphorothioate vs. a total of 90 $\mu\text{g/ml}$ for phosphodiester).

Other stabilized oligonucleotides include: nonionic DNA analogs, such as alkyl- and aryl-phosphates (in which the charged phosphonate oxygen is replaced by an alkyl or aryl group), phosphodiester and alkylphosphotriesters, in which the charged oxygen moiety is alkylated. Oligonucleotides which contain diol, such as tetraethyleneglycol or hexaethyleneglycol, at either or both termini have also been shown to be substantially resistant to nuclease degradation.

The CpG oligonucleotides useful according to the invention are S- and R-chiral CpG oligonucleotides. An "S chiral CpG oligonucleotide" as used herein is a CpG nucleic acid wherein at least two nucleotides have a backbone modification forming a chiral center and wherein a plurality of the chiral centers have S chirality. An "R chiral CpG oligonucleotide" as used herein is a CpG nucleic acid wherein at least two nucleotides have a backbone modification forming a chiral center and wherein a plurality of the chiral centers have R chirality. The backbone modification may be any type of modification that forms a chiral center. The modifications include but are not limited to phosphorothioate, methylphosphonate, methylphosphorothioate, phosphorodithioate, and combinations thereof.

The chiral CpG oligonucleotides must have at least two nucleotides within the oligonucleotide that have a backbone modification. All or less than all of the nucleotides in the oligonucleotides, however, may have a modified backbone. Of the nucleotides having a modified backbone (referred to as chiral centers), a plurality have a single chirality, S or R. A "plurality" as used herein refers to an amount greater than 50%. Thus, less than all of the chiral centers may have S or R chirality as long as a plurality of the chiral centers have S or R chirality.

In some embodiments at least 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, or 100% of the chiral centers have S or R chirality. In other embodiments at least 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, or 100% of the nucleotides have backbone modifications.

5 The S- and R- chiral CpG oligonucleotides may be prepared by any method known in the art for producing chirally pure oligonucleotides. Many references, such as the Stec et al reference cited in the Examples which teaches methods for producing stereopure phosphorothioate oligodeoxynucleotides using an oxathiaphospholane method have been published (Stec, W.J., et al., 1995, *J. Am. Chem. Soc.*, 117:12019). Other methods for making
10 chirally pure oligonucleotides have been described by companies such as ISIS Pharmaceuticals. US Patents have also described these methods. For instance U.S Patent Nos. 5883237; 5837856; 5599797; 5512668; 5856465; 5359052; 5506212; 5521302; and 5212295, each of which is hereby incorporated by reference in its entirety, disclose methods for generating stereopure oligonucleotides.

15 The S-chiral CpG oligonucleotides are useful in some aspects of the invention as a prophylactic vaccine for the treatment of a subject at risk of developing an infection with an infectious organism or a cancer in which a specific cancer antigen has been identified or an allergy or asthma where the allergen or predisposition to asthma is known. The S-chiral CpG oligonucleotides can also be given without the antigen or allergen for shorter term protection
20 against infection, allergy or cancer, and in this case repeated doses will allow longer term protection. A "subject at risk" as used herein is a subject who has any risk of exposure to an infection causing pathogen or a cancer or an allergen or a risk of developing cancer. For instance, a subject at risk may be a subject who is planning to travel to an area where a particular type of infectious agent is found or it may be a subject who through lifestyle or medical
25 procedures is exposed to bodily fluids which may contain infectious organisms or even any subject living in an area where an infectious organism or an allergen has been identified. Subjects at risk of developing infection also include general populations to which a medical agency recommends vaccination with a particular infectious organism antigen. If the antigen is an allergen and the subject develops allergic responses to that particular antigen and the subject
30 may be exposed to the antigen, i.e., during pollen season, then that subject is at risk of exposure to the antigen. A subject at risk of developing an allergy to asthma includes those subjects that have been identified as having an allergy or asthma but that don't have the active disease during

the CpG treatment as well as subjects that are considered to be at risk of developing these diseases because of genetic or environmental factors.

A subject at risk of developing a cancer is one who is who has a high probability of developing cancer. These subjects include, for instance, subjects having a genetic abnormality, the presence of which has been demonstrated to have a correlative relation to a higher likelihood of developing a cancer and subjects exposed to cancer causing agents such as tobacco, asbestos, or other chemical toxins, or a subject who has previously been treated for cancer and is in apparent remission. When a subject at risk of developing a cancer is treated with an antigen specific for the type of cancer to which the subject is at risk of developing a S-chiral CpG oligonucleotide the subject may be able to kill the cancer cells as they develop. If a tumor begins to form in the subject, the subject will develop a specific immune response against the tumor antigen.

In addition to the use of the CpG oligonucleotide for prophylactic treatment, the invention also encompasses the use of the CpG oligonucleotide for the treatment of a subject having an infection, an allergy, asthma, or a cancer.

A "subject having an infection" is a subject that has been exposed to an infectious pathogen and has acute or chronic detectable levels of the pathogen in the body. The CpG oligonucleotide can be used with an antigen to mount an antigen specific mucosal immune response that is capable of reducing the level of or eradicating the infectious pathogen. An infectious disease, as used herein, is a disease arising from the presence of a foreign microorganism in the body. It is particularly important to develop effective vaccine strategies and treatments to protect the body's mucosal surfaces, which are the primary site of pathogenic entry.

A "subject having an allergy" is a subject that has or is at risk of developing an allergic reaction in response to an allergen. An "allergy" refers to acquired hypersensitivity to a substance (allergen). Allergic conditions include but are not limited to eczema, allergic rhinitis or coryza, hay fever, conjunctivitis, bronchial asthma, urticaria (hives) and food allergies, and other atopic conditions.

Currently, allergic diseases are generally treated by the injection of small doses of antigen followed by subsequent increasing dosage of antigen. It is believed that this procedure induces tolerization to the allergen to prevent further allergic reactions. These methods, however, can

take several years to be effective and are associated with the risk of side effects such as anaphylactic shock. The methods of the invention avoid these problems.

Allergies are generally caused by IgE antibody generation against harmless allergens. The cytokines that are induced by mucosal administration of unmethylated CpG oligonucleotides are predominantly of a class called "Th1" (examples are IL-12 and IFN- γ) and these induce both humoral and cellular immune responses. The types of antibodies associated with a Th1 response are generally more protective because they have high neutralization and opsonization capabilities. The other major type of immune response, which is associated with the production of IL-4, IL-5 and IL-10 cytokines, is termed a Th2 immune response. Th2 responses involve predominately antibodies and these have less protective effect against infection and some Th2 isotypes (e.g., IgE) are associated with allergy. In general, it appears that allergic diseases are mediated by Th2 type immune responses while Th1 responses provide the best protection against infection, although excessive Th1 responses are associated with autoimmune disease. Based on the ability of the CpG oligonucleotides to shift the immune response in a subject from a Th2 (which is associated with production of IgE antibodies and allergy) to a Th1 response (which is protective against allergic reactions), an effective dose for inducing an immune response of a CpG oligonucleotide can be administered to a subject to treat or prevent an allergy.

Thus, the CpG oligonucleotide has significant therapeutic utility in the treatment of allergic and non-allergic conditions such as asthma. Th2 cytokines, especially IL-4 and IL-5 are elevated in the airways of asthmatic subjects. These cytokines promote important aspects of the asthmatic inflammatory response, including IgE isotope switching, eosinophil chemotaxis and activation and mast cell growth. Th1 cytokines, especially IFN- γ and IL-12, can suppress the formation of Th2 clones and production of Th2 cytokines. "Asthma" refers to a disorder of the respiratory system characterized by inflammation, narrowing of the airways and increased reactivity of the airways to inhaled agents. Asthma is frequently, although not exclusively associated with atopic or allergic symptoms.

A "subject having a cancer" is a subject that has detectable cancerous cells. The cancer may be a malignant or non-malignant cancer. Cancers or tumors include but are not limited to biliary tract cancer; brain cancer; breast cancer; cervical cancer; choriocarcinoma; colon cancer; endometrial cancer; esophageal cancer; gastric cancer; intraepithelial neoplasms; lymphomas; liver cancer; lung cancer (e.g. small cell and non-small cell); melanoma; neuroblastomas; oral

cancer; ovarian cancer; pancreas cancer; prostate cancer; rectal cancer; sarcomas; skin cancer; testicular cancer; thyroid cancer; and renal cancer, as well as other carcinomas and sarcomas.

A "subject" shall mean a human or vertebrate animal including but not limited to a dog, cat, horse, cow, pig, sheep, goat, chicken, primate, e.g., monkey, fish (aquaculture species), e.g. salmon, rat, and mouse.

The subject is exposed to the antigen. As used herein, the term "exposed to" refers to either the active step of contacting the subject with an antigen or the passive exposure of the subject to the antigen *in vivo*. Methods for the active exposure of a subject to an antigen are well-known in the art. In general, an antigen is administered directly to the subject by any means such as intravenous, intramuscular, oral, transdermal, mucosal, intranasal, intratracheal, or subcutaneous administration. The antigen can be administered systemically or locally. Methods for administering the antigen and the S-chiral CpG oligonucleotide are described in more detail below. A subject is passively exposed to an antigen if an antigen becomes available for exposure to the immune cells in the body. A subject may be passively exposed to an antigen, for instance, by entry of a foreign pathogen into the body or by the development of a tumor cell expressing a foreign antigen on its surface.

The methods in which a subject is passively exposed to an antigen can be particularly dependent on timing of administration of the S-chiral CpG oligonucleotide. For instance, in a subject at risk of developing a cancer or an infectious disease or an allergic or asthmatic response, the subject may be administered the S-chiral CpG oligonucleotide on a regular basis when that risk is greatest, i.e., during allergy season or after exposure to a cancer causing agent. Additionally the CpG oligonucleotide may be administered to travelers before they travel to foreign lands where they are at risk of exposure to infectious agents. Likewise the S-chiral CpG oligonucleotide may be administered to soldiers or civilians at risk of exposure to biowarfare to induce a mucosal immune response to the antigen when and if the subject is exposed to it.

An "antigen" as used herein is a molecule capable of provoking an immune response. Antigens include but are not limited to cells, cell extracts, proteins, polypeptides, peptides, polysaccharides, polysaccharide conjugates, peptide and non-peptide mimics of polysaccharides and other molecules, small molecules, lipids, glycolipids, carbohydrates, viruses and viral extracts and multicellular organisms such as parasites and allergens. The term antigen broadly includes any type of molecule which is recognized by a host immune system as being foreign. Antigens include but are not limited to cancer antigens, microbial antigens, and allergens.

A "cancer antigen" as used herein is a compound, such as a peptide or protein, associated with a tumor or cancer cell surface and which is capable of provoking an immune response when expressed on the surface of an antigen presenting cell in the context of an MHC molecule.

Cancer antigens can be prepared from cancer cells either by preparing crude extracts of cancer cells, for example, as described in Cohen, et al., 1994, *Cancer Research*, 54:1055, by partially purifying the antigens, by recombinant technology, or by de novo synthesis of known antigens. Cancer antigens include but are not limited to antigens that are recombinantly expressed, an immunogenic portion of, or a whole tumor or cancer. Such antigens can be isolated or prepared recombinantly or by any other means known in the art.

10 A "microbial antigen" as used herein is an antigen of a microorganism and includes but is not limited to virus, bacteria, parasites, and fungi. Such antigens include the intact microorganism as well as natural isolates and fragments or derivatives thereof and also synthetic compounds which are identical to or similar to natural microorganism antigens and induce an immune response specific for that microorganism. A compound is similar to a natural
15 microorganism antigen if it induces an immune response (humoral and/or cellular) to a natural microorganism antigen. Such antigens are used routinely in the art and are well known to those of ordinary skill in the art.

Examples of virus that have been found in humans include but are not limited to:

Retroviridae (e.g. human immunodeficiency viruses, such as HIV-1 (also referred to as HTLV-III, LAV or HTLV-III/LAV, or HIV-III; and other isolates, such as HIV-LP; *Picornaviridae* (e.g. polio viruses, hepatitis A virus; enteroviruses, human Coxsackie viruses, rhinoviruses, echoviruses); *Calciviridae* (e.g. strains that cause gastroenteritis); *Togaviridae* (e.g. equine encephalitis viruses, rubella viruses); *Flaviridae* (e.g. dengue viruses, encephalitis viruses, yellow fever viruses); *Coronaviridae* (e.g. coronaviruses); *Rhabdoviridae* (e.g. vesicular
25 stomatitis viruses, rabies viruses); *Coronaviridae* (e.g. coronaviruses); *Rhabdoviridae* (e.g. vesicular stomatitis viruses, rabies viruses); *Filoviridae* (e.g. ebola viruses); *Paramyxoviridae* (e.g. parainfluenza viruses, mumps virus, measles virus, respiratory syncytial virus); *Orthomyxoviridae* (e.g. influenza viruses); *Bungaviridae* (e.g. Hantaan viruses, bunga viruses, phleboviruses and Nairo viruses); *Arenaviridae* (hemorrhagic fever viruses); *Reoviridae* (e.g.
30 reoviruses, orbiviruses and rotaviruses); *Birnaviridae*; *Hepadnaviridae* (Hepatitis B virus); *Parvoviridae* (parvoviruses); *Papovaviridae* (papilloma viruses, polyoma viruses); *Adenoviridae* (most adenoviruses); *Herpesviridae* (herpes simplex virus (HSV) 1 and 2, varicella zoster virus,

cytomegalovirus (CMV), herpes virus; *Poxviridae* (variola viruses, vaccinia viruses, pox viruses); and *Iridoviridae* (e.g. African swine fever virus); and unclassified viruses (e.g. the etiological agents of Spongiform encephalopathies, the agent of delta hepatitis (thought to be a defective satellite of hepatitis B virus), the agents of non-A, non-B hepatitis (class 1 = internally transmitted; class 2 = parenterally transmitted (i.e. Hepatitis C); Norwalk and related viruses, and astroviruses).

Both gram negative and gram positive bacteria serve as antigens in vertebrate animals. Such gram positive bacteria include, but are not limited to *Pasteurella* species, *Staphylococci* species, and *Streptococcus* species. Gram negative bacteria include, but are not limited to, *Escherichia coli*, *Pseudomonas* species, and *Salmonella* species. Specific examples of infectious bacteria include but are not limited to: *Helicobacter pylori*, *Borrelia burgdorferi*, *Legionella pneumophila*, *Mycobacteria* sps (e.g. *M. tuberculosis*, *M. avium*, *M. intracellulare*, *M. kansasii*, *M. gordonae*), *Staphylococcus aureus*, *Neisseria gonorrhoeae*, *Neisseria meningitidis*, *Listeria monocytogenes*, *Streptococcus pyogenes* (Group A Streptococcus), *Streptococcus agalactiae* (Group B Streptococcus), *Streptococcus* (viridans group), *Streptococcus faecalis*, *Streptococcus bovis*, *Streptococcus* (anaerobic sps.), *Streptococcus pneumoniae*, pathogenic *Campylobacter* sp., *Enterococcus* sp., *Haemophilus influenzae*, *Bacillus anthracis*, *Corynebacterium diphtheriae*, *Corynebacterium* sp., *Erysipelothrix rhusiopathiae*, *Clostridium perfringens*, *Clostridium tetani*, *Enterobacter aerogenes*, *Klebsiella pneumoniae*, *Pasteurella multocida*, *Bacteroides* sp., *Fusobacterium nucleatum*, *Streptobacillus moniliformis*, *Treponema pallidum*, *Treponema pertenue*, *Leptospira*, *Rickettsia*, and *Actinomyces israelii*.

Examples of fungi include: *Cryptococcus neoformans*, *Histoplasma capsulatum*, *Coccidioides immitis*, *Blastomyces dermatitidis*, *Chlamydia trachomatis*, *Candida albicans*. Other infectious organisms (i.e., protists) include: *Plasmodium* such as *Plasmodium falciparum*, *Plasmodium malariae*, *Plasmodium ovale*, and *Plasmodium vivax* and *Toxoplasma gondii*.

Other medically relevant microorganisms have been described extensively in the literature, e.g., see C.G.A Thomas, *Medical Microbiology*, Bailliere Tindall, Great Britain 1983, the entire contents of which is hereby incorporated by reference.

Although many of the microbial antigens described above relate to human disorders, the invention is also useful for treating other nonhuman vertebrates. Nonhuman vertebrates are also capable of developing infections which can be prevented or treated with the CpG

oligonucleotides disclosed herein. For instance, in addition to the treatment of infectious human diseases, the methods of the invention are useful for treating infections of animals.

As used herein, the term "treat", "treated", or "treating" when used with respect to an infectious disease refers to a prophylactic treatment which increases the resistance of a subject (a subject at risk of infection) to infection with a pathogen or, in other words, decreases the likelihood that the subject will become infected with the pathogen as well as a treatment after the subject (a subject who has been infected) has become infected in order to fight the infection, e.g., reduce or eliminate the infection or prevent it from becoming worse.

Many vaccines for the treatment of non-human vertebrates are disclosed in Bennett, K. *Compendium of Veterinary Products*, 3rd ed. North American Compendiums, Inc., 1995. As discussed above, antigens include infectious microbes such as virus, bacteria and fungi and fragments thereof, derived from natural sources or synthetically. Infectious virus of both human and non-human vertebrates, include retroviruses, RNA viruses and DNA viruses. This group of retroviruses includes both simple retroviruses and complex retroviruses. The simple retroviruses include the subgroups of B-type retroviruses, C-type retroviruses and D-type retroviruses. An example of a B-type retrovirus is mouse mammary tumor virus (MMTV). The C-type retroviruses include subgroups C-type group A (including Rous sarcoma virus (RSV), avian leukemia virus (ALV), and avian myeloblastosis virus (AMV)) and C-type group B (including murine leukemia virus (MLV), feline leukemia virus (FeLV), murine sarcoma virus (MSV), gibbon ape leukemia virus (GALV), spleen necrosis virus (SNV), reticuloendotheliosis virus (RV) and simian sarcoma virus (SSV)). The D-type retroviruses include Mason-Pfizer monkey virus (MPMV) and simian retrovirus type 1 (SRV-1). The complex retroviruses include the subgroups of lentiviruses, T-cell leukemia viruses and the foamy viruses. Lentiviruses include HIV-1, but also include HIV-2, SIV, Visna virus, feline immunodeficiency virus (FIV), and equine infectious anemia virus (EIAV). The T-cell leukemia viruses include HTLV-1, HTLV-II, simian T-cell leukemia virus (STLV), and bovine leukemia virus (BLV). The foamy viruses include human foamy virus (HFV), simian foamy virus (SFV) and bovine foamy virus (BFV).

Examples of other RNA viruses that are antigens in vertebrate animals include, but are not limited to, the following: members of the family Reoviridae, including the genus Orthoreovirus (multiple serotypes of both mammalian and avian retroviruses), the genus Orbivirus (Bluetongue virus, Eugenangee virus, Kemerovo virus, African horse sickness virus,

and Colorado Tick Fever virus), the genus Rotavirus (human rotavirus, Nebraska calf diarrhea virus, murine rotavirus, simian rotavirus, bovine or ovine rotavirus, avian rotavirus); the family Picornaviridae, including the genus Enterovirus (poliovirus, Coxsackie virus A and B, enteric cytopathic human orphan (ECHO) viruses, hepatitis A virus, Simian enteroviruses, Murine

5 encephalomyelitis (ME) viruses, Poliovirus muris, Bovine enteroviruses, Porcine enteroviruses, the genus Cardiovirus (Encephalomyocarditis virus (EMC), Mengovirus), the genus Rhinovirus (Human rhinoviruses including at least 113 subtypes; other rhinoviruses), the genus Aphovirus (Foot and Mouth disease (FMDV); the family Calciviridae, including Vesicular exanthema of swine virus, San Miguel sea lion virus, Feline picornavirus and Norwalk virus; the family

10 Togaviridae, including the genus Alphavirus (Eastern equine encephalitis virus, Semliki forest virus, Sindbis virus, Chikungunya virus, O'Nyong-Nyong virus, Ross river virus, Venezuelan equine encephalitis virus, Western equine encephalitis virus), the genus Flavivirus (Mosquito borne yellow fever virus, Dengue virus, Japanese encephalitis virus, St. Louis encephalitis virus, Murray Valley encephalitis virus, West Nile virus, Kunjin virus, Central European tick borne

15 virus, Far Eastern tick borne virus, Kyasanur forest virus, Louping III virus, Powassan virus, Omsk hemorrhagic fever virus), the genus Rubivirus (Rubella virus), the genus Pestivirus (Mucosal disease virus, Hog cholera virus, Border disease virus); the family Bunyaviridae, including the genus Bunyavirus (Bunyamwera and related viruses, California encephalitis group viruses), the genus Phlebovirus (Sandfly fever Sicilian virus, Rift Valley fever virus), the genus

20 Nairovirus (Crimean-Congo hemorrhagic fever virus, Nairobi sheep disease virus), and the genus Uukuvirus (Uukuniemi and related viruses); the family Orthomyxoviridae, including the genus Influenza virus (Influenza virus type A, many human subtypes); Swine influenza virus, and Avian and Equine Influenza viruses; influenza type B (many human subtypes), and influenza type C (possible separate genus); the family paramyxoviridae, including the genus

25 Paramyxovirus (Parainfluenza virus type 1, Sendai virus, Hemadsorption virus, Parainfluenza viruses types 2 to 5, Newcastle Disease Virus, Mumps virus), the genus Morbillivirus (Measles virus, subacute sclerosing panencephalitis virus, distemper virus, Rinderpest virus), the genus Pneumovirus (respiratory syncytial virus (RSV), Bovine respiratory syncytial virus and Pneumonia virus of mice); forest virus, Sindbis virus, Chikungunya virus, O'Nyong-Nyong virus,

30 Ross river virus, Venezuelan equine encephalitis virus, Western equine encephalitis virus), the genus Flavivirus (Mosquito borne yellow fever virus, Dengue virus, Japanese encephalitis virus, St. Louis encephalitis virus, Murray Valley encephalitis virus, West Nile virus, Kunjin virus,

Central European tick borne virus, Far Eastern tick borne virus, Kyasanur forest virus, Louping
 III virus, Powassan virus, Omsk hemorrhagic fever virus), the genus Rubivirus (Rubella virus),
 the genus Pestivirus (Mucosal disease virus, Hog cholera virus, Border disease virus); the family
 Bunyaviridae, including the genus Bunyvirus (Bunyamwera and related viruses, California
 5 encephalitis group viruses), the genus Phlebovirus (Sandfly fever Sicilian virus, Rift Valley fever
 virus), the genus Nairovirus (Crimean-Congo hemorrhagic fever virus, Nairobi sheep disease
 virus), and the genus Uukuvirus (Uukuniemi and related viruses); the family Orthomyxoviridae,
 including the genus Influenza virus (Influenza virus type A, many human subtypes); Swine
 influenza virus, and Avian and Equine Influenza viruses; influenza type B (many human
 10 subtypes), and influenza type C (possible separate genus); the family paramyxoviridae,
 including the genus Paramyxovirus (Parainfluenza virus type 1, Sendai virus, Hemadsorption
 virus, Parainfluenza viruses types 2 to 5, Newcastle Disease Virus, Mumps virus), the genus
 Morbillivirus (Measles virus, subacute sclerosing panencephalitis virus, distemper virus,
 Rinderpest virus), the genus Pneumovirus (respiratory syncytial virus (RSV), Bovine respiratory
 15 syncytial virus and Pneumonia virus of mice); the family Rhabdoviridae, including the genus
 Vesiculovirus (VSV), Chandipura virus, Flanders-Hart Park virus), the genus Lyssavirus (Rabies
 virus), fish Rhabdoviruses, and two probable Rhabdoviruses (Marburg virus and Ebola virus);
 the family Arenaviridae, including Lymphocytic choriomeningitis virus (LCM), Tacaribe virus
 complex, and Lassa virus; the family Coronaviridae, including Infectious Bronchitis Virus
 20 (IBV), Mouse Hepatitis virus, Human enteric corona virus, and Feline infectious peritonitis
 (Feline coronavirus).

Illustrative DNA viruses that are antigens in vertebrate animals include, but are not
 limited to: the family Poxviridae, including the genus Orthopoxvirus (Variola major, Variola
 minor, Monkey pox Vaccinia, Cowpox, Buffalopox, Rabbitpox, Ectromelia), the genus
 25 Leporipoxvirus (Myxoma, Fibroma), the genus Avipoxvirus (Fowlpox, other avian poxvirus),
 the genus Capripoxvirus (sheeppox, goatpox), the genus Suipoxvirus (Swinepox), the genus
 Parapoxvirus (contagious postular dermatitis virus, pseudocowpox, bovine papular stomatitis
 virus); the family Iridoviridae (African swine fever virus, Frog viruses 2 and 3, Lymphocystis
 virus of fish); the family Herpesviridae, including the alpha-Herpesviruses (Herpes Simplex
 30 Types 1 and 2, Varicella-Zoster, Equine abortion virus, Equine herpes virus 2 and 3,
 pseudorabies virus, infectious bovine keratoconjunctivitis virus, infectious bovine rhinotracheitis
 virus, feline rhinotracheitis virus, infectious laryngotracheitis virus) the Beta-herpesviruses

(Human cytomegalovirus and cytomegaloviruses of swine, monkeys and rodents); the gamma-herpesviruses (Epstein-Barr virus (EBV), Marek's disease virus, Herpes saimiri, Herpesvirus ateles, Herpesvirus sylvilagus, guinea pig herpes virus, Lucke tumor virus); the family Adenoviridae, including the genus Mastadenovirus (Human subgroups A,B,C,D,E and ungrouped; simian adenoviruses (at least 23 serotypes), infectious canine hepatitis, and adenoviruses of cattle, pigs, sheep, frogs and many other species, the genus Aviadenovirus (Avian adenoviruses); and non-cultivable adenoviruses; the family Papoviridae, including the genus Papillomavirus (Human papilloma viruses, bovine papilloma viruses, Shope rabbit papilloma virus, and various pathogenic papilloma viruses of other species), the genus Polyomavirus (polyomavirus, Simian vacuolating agent (SV-40), Rabbit vacuolating agent (RVK), K virus, BK virus, JC virus, and other primate polyoma viruses such as Lymphotropic papilloma virus); the family Parvoviridae including the genus Adeno-associated viruses, the genus Parvovirus (Feline panleukopenia virus, bovine parvovirus, canine parvovirus, Aleutian mink disease virus, etc). Finally, DNA viruses may include viruses which do not fit into the above families such as Kuru and Creutzfeldt-Jacob disease viruses and chronic infectious neuropathic agents (CHINA virus).

Each of the foregoing lists is illustrative, and is not intended to be limiting.

In addition to the use of the S-chiral CpG oligonucleotides to induce an antigen specific immune response in humans, the methods of the preferred embodiments are particularly well suited for treatment of birds such as hens, chickens, turkeys, ducks, geese, quail, and pheasant. Birds are prime targets for many types of infections.

Hatching birds are exposed to pathogenic microorganisms shortly after birth. Although these birds are initially protected against pathogens by maternal derived antibodies, this protection is only temporary, and the bird's own immature immune system must begin to protect the bird against the pathogens. It is often desirable to prevent infection in young birds when they are most susceptible. It is also desirable to prevent against infection in older birds, especially when the birds are housed in closed quarters, leading to the rapid spread of disease. Thus, it is desirable to administer the CpG oligonucleotide and the non-nucleic acid adjuvant of the invention to birds to enhance an antigen-specific immune response when antigen is present.

An example of a common infection in chickens is chicken infectious anemia virus (CIAV). CIAV was first isolated in Japan in 1979 during an investigation of a Marek's disease vaccination break (Yuasa et al., 1979, Avian Dis. 23:366-385). Since that time, CIAV has been

detected in commercial poultry in all major poultry producing countries (van Bulow et al., 1991, pp.690-699) in Diseases of Poultry, 9th edition, Iowa State University Press).

CIAV infection results in a clinical disease, characterized by anemia, hemorrhage and immunosuppression, in young susceptible chickens. Atrophy of the thymus and of the bone marrow and consistent lesions of CIAV-infected chickens are also characteristic of CIAV infection. Lymphocyte depletion in the thymus, and occasionally in the bursa of Fabricius, results in immunosuppression and increased susceptibility to secondary viral, bacterial, or fungal infections which then complicate the course of the disease. The immunosuppression may cause aggravated disease after infection with one or more of Marek's disease virus (MDV), infectious bursal disease virus, reticuloendotheliosis virus, adenovirus, or reovirus. It has been reported that pathogenesis of MDV is enhanced by CIAV (DeBoer et al., 1989, p. 28 In Proceedings of the 38th Western Poultry Diseases Conference, Tempe, Ariz.). Further, it has been reported that CIAV aggravates the signs of infectious bursal disease (Rosenberger et al., 1989, Avian Dis. 33:707-713). Chickens develop an age resistance to experimentally induced disease due to CAA. This is essentially complete by the age of 2 weeks, but older birds are still susceptible to infection (Yuasa, N. et al., 1979 supra; Yuasa, N. et al., Avian Diseases 24, 202-209, 1980). However, if chickens are dually infected with CAA and an immunosuppressive agent (IBDV, MDV etc.) age resistance against the disease is delayed (Yuasa, N. et al., 1979 and 1980 supra; Bulow von V. et al., J. Veterinary Medicine 33, 93-116, 1986). Characteristics of CIAV that may potentiate disease transmission include high resistance to environmental inactivation and some common disinfectants. The economic impact of CIAV infection on the poultry industry is clear from the fact that 10% to 30% of infected birds in disease outbreaks die.

Vaccination of birds, like other vertebrate animals can be performed at any age. Normally, vaccinations are performed at up to 12 weeks of age for a live microorganism and between 14-18 weeks for an inactivated microorganism or other type of vaccine. For in ovo vaccination, vaccination can be performed in the last quarter of embryo development. The vaccine may be administered subcutaneously, by spray, orally, intraocularly, intratracheally, nasally, or by other mucosal delivery methods described herein. Thus, the CpG oligonucleotide of the invention can be administered to birds and other non-human vertebrates using routine vaccination schedules and the antigen is administered after an appropriate time period as described herein.

Cattle and livestock are also susceptible to infection. Disease which affect these animals can produce severe economic losses, especially amongst cattle. The methods of the invention can be used to protect against infection in livestock, such as cows, horses, pigs, sheep, and goats.

Cows can be infected by bovine viruses. Bovine viral diarrhea virus (BVDV) is a small
5 enveloped positive-stranded RNA virus and is classified, along with hog cholera virus (HOCV) and sheep border disease virus (BDV), in the pestivirus genus. Although, Pestiviruses were previously classified in the Togaviridae family, some studies have suggested their reclassification within the Flaviviridae family along with the flavivirus and hepatitis C virus (HCV) groups (Francki, et al., 1991).

10 BVDV, which is an important pathogen of cattle can be distinguished, based on cell culture analysis, into cytopathogenic (CP) and noncytopathogenic (NCP) biotypes. The NCP biotype is more widespread although both biotypes can be found in cattle. If a pregnant cow becomes infected with an NCP strain, the cow can give birth to a persistently infected and specifically immunotolerant calf that will spread virus during its lifetime. The persistently
15 infected cattle can succumb to mucosal disease and both biotypes can then be isolated from the animal. Clinical manifestations can include abortion, teratogenesis, and respiratory problems, mucosal disease and mild diarrhea. In addition, severe thrombocytopenia, associated with herd epidemics, that may result in the death of the animal has been described and strains associated with this disease seem more virulent than the classical BVDVs.

20 Equine herpesviruses (EHV) comprise a group of antigenically distinct biological agents which cause a variety of infections in horses ranging from subclinical to fatal disease. These include Equine herpesvirus-1 (EHV-1), a ubiquitous pathogen in horses. EHV-1 is associated with epidemics of abortion, respiratory tract disease, and central nervous system disorders. Primary infection of upper respiratory tract of young horses results in a febrile illness which lasts
25 for 8 to 10 days. Immunologically experienced mares may be reinfected via the respiratory tract without disease becoming apparent, so that abortion usually occurs without warning. The neurological syndrome is associated with respiratory disease or abortion and can affect animals of either sex at any age, leading to incoordination, weakness and posterior paralysis (Telford, E. A. R. et al., Virology 189, 304-316, 1992). Other EHV's include EHV-2, or equine
30 cytomegalovirus, EHV-3, equine coital exanthema virus, and EHV-4, previously classified as EHV-1 subtype 2.

Sheep and goats can be infected by a variety of dangerous microorganisms including visna-maedi.

Primates such as monkeys, apes and macaques can be infected by simian immunodeficiency virus. Inactivated cell-virus and cell-free whole simian immunodeficiency vaccines have been reported to afford protection in macaques (Stott et al. (1990) Lancet 36:1538-1541; Desrosiers et al. PNAS USA (1989) 86:6353-6357; Murphey-Corb et al. (1989) Science 246:1293-1297; and Carlson et al. (1990) AIDS Res. Human Retroviruses 6:1239-1246). A recombinant HIV gp120 vaccine has been reported to afford protection in chimpanzees (Berman et al. (1990) Nature 345:622-625).

10 Cats, both domestic and wild, are susceptible to infection with a variety of microorganisms. For instance, feline infectious peritonitis is a disease which occurs in both domestic and wild cats, such as lions, leopards, cheetahs, and jaguars. When it is desirable to prevent infection with this and other types of pathogenic organisms in cats, the methods of the invention can be used to vaccinate cats to protect them against infection.

15 Domestic cats may become infected with several retroviruses, including but not limited to feline leukemia virus (FeLV), feline sarcoma virus (FeSV), endogenous type C oncornavirus (RD-114), and feline syncytia-forming virus (FeSFV). Of these, FeLV is the most significant pathogen, causing diverse symptoms, including lymphoreticular and myeloid neoplasms, anemias, immune mediated disorders, and an immunodeficiency syndrome which is similar to
20 human acquired immune deficiency syndrome (AIDS). Recently, a particular replication-defective FeLV mutant, designated FeLV-AIDS, has been more particularly associated with immunosuppressive properties.

The discovery of feline T-lymphotropic lentivirus (also referred to as feline immunodeficiency) was first reported in Pedersen et al. (1987) Science 235:790-793.
25 Characteristics of FIV have been reported in Yamamoto et al. (1988) Leukemia, December Supplement 2:204S-215S; Yamamoto et al. (1988) Am. J. Vet. Res. 49:1246-1258; and Ackley et al. (1990) J. Virol. 64:5652-5655. Cloning and sequence analysis of FIV have been reported in Olmsted et al. (1989) Proc. Natl. Acad. Sci. USA 86:2448-2452 and 86:4355-4360.

Feline infectious peritonitis (FIP) is a sporadic disease occurring unpredictably in
30 domestic and wild Felidae. While FIP is primarily a disease of domestic cats, it has been diagnosed in lions, mountain lions, leopards, cheetahs, and the jaguar. Smaller wild cats that have been afflicted with FIP include the lynx and caracal, sand cat, and pallas cat. In domestic

cats, the disease occurs predominantly in young animals, although cats of all ages are susceptible. A peak incidence occurs between 6 and 12 months of age. A decline in incidence is noted from 5 to 13 years of age, followed by an increased incidence in cats 14 to 15 years old.

Viral, bacterial, and parasitic diseases in fin-fish, shellfish or other aquatic life forms pose a serious problem for the aquaculture industry. Owing to the high density of animals in the hatchery tanks or enclosed marine farming areas, infectious diseases may eradicate a large proportion of the stock in, for example, a fin-fish, shellfish, or other aquatic life forms facility. Prevention of disease is a more desired remedy to these threats to fish than intervention once the disease is in progress. Vaccination of fish is the only preventative method which may offer long-term protection through immunity. Nucleic acid based vaccinations are described in US Patent No. 5,780,448 issued to Davis.

The fish immune system has many features similar to the mammalian immune system, such as the presence of B cells, T cells, lymphokines, complement, and immunoglobulins. Fish have lymphocyte subclasses with roles that appear similar in many respects to those of the B and T cells of mammals. Vaccines can be administered by immersion or orally.

Aquaculture species include but are not limited to fin-fish, shellfish, and other aquatic animals. Fin-fish include all vertebrate fish, which may be bony or cartilaginous fish, such as, for example, salmonids, carp, catfish, yellowtail, seabream, and seabass. Salmonids are a family of fin-fish which include trout (including rainbow trout), salmon, and Arctic char. Examples of shellfish include, but are not limited to, clams, lobster, shrimp, crab, and oysters. Other cultured aquatic animals include, but are not limited to eels, squid, and octopi.

Polypeptides of viral aquaculture pathogens include but are not limited to glycoprotein (G) or nucleoprotein (N) of viral hemorrhagic septicemia virus (VHSV); G or N proteins of infectious hematopoietic necrosis virus (IHNV); VP1, VP2, VP3 or N structural proteins of infectious pancreatic necrosis virus (IPNV); G protein of spring viremia of carp (SVC); and a membrane-associated protein, tegumin or capsid protein or glycoprotein of channel catfish virus (CCV).

Polypeptides of bacterial pathogens include but are not limited to an iron-regulated outer membrane protein, (IROMP), an outer membrane protein (OMP), and an A-protein of *Aeromonis salmonicida* which causes furunculosis, p57 protein of *Renibacterium salmoninarum* which causes bacterial kidney disease (BKD), major surface associated antigen (msa), a surface expressed cytotoxin (mpr), a surface expressed hemolysin (ish), and a flagellar antigen of

- Yersiniosis; an extracellular protein (ECP), an iron-regulated outer membrane protein (IROMP), and a structural protein of Pasteurellosis; an OMP and a flagellar protein of Vibrosis anguillarum and *V. ordalii*; a flagellar protein, an OMP protein, *aroA*, and *purA* of Edwardsiellosis ictaluri and *E. tarda*; and surface antigen of Ichthyophthirius; and a structural and regulatory protein of
- 5 Cytophaga columnari; and a structural and regulatory protein of Rickettsia.

Polypeptides of a parasitic pathogen include but are not limited to the surface antigens of Ichthyophthirius.

- An "allergen" refers to a substance (antigen) that can induce an allergic or asthmatic response in a susceptible subject. The list of allergens is enormous and can include pollens,
- 10 insect venoms, animal dander dust, fungal spores and drugs (e.g. penicillin). Examples of natural, animal and plant allergens include but are not limited to proteins specific to the following genres: *Canine* (*Canis familiaris*); *Dermatophagoides* (e.g. *Dermatophagoides farinae*); *Felis* (*Felis domesticus*); *Ambrosia* (*Ambrosia artemisiifolia*; *Lolium* (e.g. *Lolium perenne* or *Lolium multiflorum*); *Cryptomeria* (*Cryptomeria japonica*); *Alternaria* (*Alternaria alternata*);
- 15 *Alder*; *Alnus* (*Alnus gultinoasa*); *Betula* (*Betula verrucosa*); *Quercus* (*Quercus alba*); *Olea* (*Olea europa*); *Artemisia* (*Artemisia vulgaris*); *Plantago* (e.g. *Plantago lanceolata*); *Parietaria* (e.g. *Parietaria officinalis* or *Parietaria judaica*); *Blattella* (e.g. *Blattella germanica*); *Apis* (e.g. *Apis multiflorum*); *Cupressus* (e.g. *Cupressus sempervirens*, *Cupressus arizonica* and *Cupressus macrocarpa*); *Juniperus* (e.g. *Juniperus sabinoidea*, *Juniperus virginiana*, *Juniperus communis* and *Juniperus ashei*); *Thuja* (e.g. *Thuja orientalis*); *Chamaecyparis* (e.g. *Chamaecyparis obtusa*); *Periplaneta* (e.g. *Periplaneta americana*); *Agropyron* (e.g. *Agropyron repens*); *Secale* (e.g. *Secale cereale*); *Triticum* (e.g. *Triticum aestivum*); *Dactylis* (e.g. *Dactylis glomerata*); *Festuca* (e.g. *Festuca elatior*); *Poa* (e.g. *Poa pratensis* or *Poa compressa*); *Avena* (e.g. *Avena sativa*); *Holcus* (e.g. *Holcus lanatus*); *Anthoxanthum* (e.g. *Anthoxanthum odoratum*);
- 25 *Arrhenatherum* (e.g. *Arrhenatherum elatius*); *Agrostis* (e.g. *Agrostis alba*); *Phleum* (e.g. *Phleum pratense*); *Phalaris* (e.g. *Phalaris arundinacea*); *Paspalum* (e.g. *Paspalum notatum*); *Sorghum* (e.g. *Sorghum halepensis*); and *Bromus* (e.g. *Bromus inermis*).

- The antigen may be an antigen that is encoded by a nucleic acid vector or it may be not encoded in a nucleic acid vector. In the former case the nucleic acid vector is administered to the
- 30 subject and the antigen is expressed *in vivo*. In the latter case the antigen may be administered directly to the subject. An "antigen not encoded in a nucleic acid vector" as used herein refers to any type of antigen that is not a nucleic acid. For instance, in some aspects of the invention the

antigen not encoded in a nucleic acid vector is a polypeptide. Minor modifications of the primary amino acid sequences of polypeptide antigens may also result in a polypeptide which has substantially equivalent antigenic activity as compared to the unmodified counterpart polypeptide. Such modifications may be deliberate, as by site-directed mutagenesis, or may be spontaneous. All of the polypeptides produced by these modifications are included herein as long as antigenicity still exists. The polypeptide may be, for example, a viral polypeptide.

The term "substantially purified" as used herein refers to a polypeptide which is substantially free of other proteins, lipids, carbohydrates or other materials with which it is naturally associated. One skilled in the art can purify viral or bacterial polypeptides using standard techniques for protein purification. The substantially pure polypeptide will often yield a single major band on a non-reducing polyacrylamide gel. In the case of partially glycosylated polypeptides or those that have several start codons, there may be several bands on a non-reducing polyacrylamide gel, but these will form a distinctive pattern for that polypeptide. The purity of the viral or bacterial polypeptide can also be determined by amino-terminal amino acid sequence analysis. Other types of antigens not encoded by a nucleic acid vector such as polysaccharides, small molecule, mimics etc are described above.

The invention also utilizes polynucleotides encoding the antigenic polypeptides. It is envisioned that the antigen may be delivered to the subject in a nucleic acid molecule which encodes for the antigen such that the antigen must be expressed *in vivo*. Such antigens delivered to the subject in a nucleic acid vector are referred to as "antigens encoded by a nucleic acid vector." The nucleic acid encoding the antigen is operatively linked to a gene expression sequence which directs the expression of the antigen nucleic acid within a eukaryotic cell. The "gene expression sequence" is any regulatory nucleotide sequence, such as a promoter sequence or promoter-enhancer combination, which facilitates the efficient transcription and translation of the antigen nucleic acid to which it is operatively linked. The gene expression sequence may, for example, be a mammalian or viral promoter, such as a constitutive or inducible promoter. Constitutive mammalian promoters include, but are not limited to, the promoters for the following genes: hypoxanthine phosphoribosyl transferase (HPTR), adenosine deaminase, pyruvate kinase, β -actin promoter and other constitutive promoters. Exemplary viral promoters which function constitutively in eukaryotic cells include, for example, promoters from the cytomegalovirus (CMV), simian virus (e.g., SV40), papilloma virus, adenovirus, human immunodeficiency virus (HIV), Rous sarcoma virus, cytomegalovirus, the long terminal repeats

(LTR) of Moloney leukemia virus and other retroviruses, and the thymidine kinase promoter of herpes simplex virus. Other constitutive promoters are known to those of ordinary skill in the art. The promoters useful as gene expression sequences of the invention also include inducible promoters. Inducible promoters are expressed in the presence of an inducing agent. For
5 example, the metallothionein promoter is induced to promote transcription and translation in the presence of certain metal ions. Other inducible promoters are known to those of ordinary skill in the art.

In general, the gene expression sequence shall include, as necessary, 5' non-transcribing and 5' non-translating sequences involved with the initiation of transcription and translation,
10 respectively, such as a TATA box, capping sequence, CAAT sequence, and the like. Especially, such 5' non-transcribing sequences will include a promoter region which includes a promoter sequence for transcriptional control of the operably joined antigen nucleic acid. The gene expression sequences optionally include enhancer sequences or upstream activator sequences as desired.

15 The antigen nucleic acid is operatively linked to the gene expression sequence. As used herein, the antigen nucleic acid sequence and the gene expression sequence are said to be "operably linked" when they are covalently linked in such a way as to place the expression or transcription and/or translation of the antigen coding sequence under the influence or control of the gene expression sequence. Two DNA sequences are said to be operably linked if induction
20 of a promoter in the 5' gene expression sequence results in the transcription of the antigen sequence and if the nature of the linkage between the two DNA sequences does not (1) result in the introduction of a frame-shift mutation, (2) interfere with the ability of the promoter region to direct the transcription of the antigen sequence, or (3) interfere with the ability of the corresponding RNA transcript to be translated into a protein. Thus, a gene expression sequence
25 would be operably linked to an antigen nucleic acid sequence if the gene expression sequence were capable of effecting transcription of that antigen nucleic acid sequence such that the resulting transcript is translated into the desired protein or polypeptide.

The antigen nucleic acid of the invention may be delivered to the immune system alone or in association with a vector. In its broadest sense, a "vector" is any vehicle capable of
30 facilitating the transfer of the antigen nucleic acid to the cells of the immune system so that the antigen can be expressed and presented on the surface of the immune cell. The vector generally transports the nucleic acid to the immune cells with reduced degradation relative to the extent of

degradation that would result in the absence of the vector. The vector optionally includes the above-described gene expression sequence to enhance expression of the antigen nucleic acid in immune cells. In general, the vectors useful in the invention include, but are not limited to, plasmids, phagemids, viruses, other vehicles derived from viral or bacterial sources that have been manipulated by the insertion or incorporation of the antigen nucleic acid sequences. Viral vectors are a preferred type of vector and include, but are not limited to nucleic acid sequences from the following viruses: retrovirus, such as Moloney murine leukemia virus, Harvey murine sarcoma virus, murine mammary tumor virus, and Rous sarcoma virus; adenovirus, adeno-associated virus; SV40-type viruses; polyoma viruses; Epstein-Barr viruses; papilloma viruses; herpes virus; vaccinia virus; polio virus; and RNA virus such as a retrovirus. One can readily employ other vectors not named but known to the art.

Preferred viral vectors are based on non-cytopathic eukaryotic viruses in which non-essential genes have been replaced with the gene of interest. Non-cytopathic viruses include retroviruses, the life cycle of which involves reverse transcription of genomic viral RNA into DNA with subsequent proviral integration into host cellular DNA. Retroviruses have been approved for human gene therapy trials. Most useful are those retroviruses that are replication-deficient (i.e., capable of directing synthesis of the desired proteins, but incapable of manufacturing an infectious particle). Such genetically altered retroviral expression vectors have general utility for the high-efficiency transduction of genes *in vivo*. Standard protocols for producing replication-deficient retroviruses (including the steps of incorporation of exogenous genetic material into a plasmid, transfection of a packaging cell lined with plasmid, production of recombinant retroviruses by the packaging cell line, collection of viral particles from tissue culture media, and infection of the target cells with viral particles) are provided in Kriegler, M., "Gene Transfer and Expression, A Laboratory Manual" W.H. Freeman C.O., New York (1990) and Murry, E.J. "Methods in Molecular Biology", vol. 7, Humana Press, Inc., Clifton, New Jersey (1991).

A preferred virus for certain applications is the adeno-associated virus, a double-stranded DNA virus. The adeno-associated virus can be engineered to be replication -deficient and is capable of infecting a wide range of cell types and species. It further has advantages such as, heat and lipid solvent stability; high transduction frequencies in cells of diverse lineages, including hemopoietic cells; and lack of superinfection inhibition thus allowing multiple series of transductions. Reportedly, the adeno-associated virus can integrate into human cellular DNA in

a site-specific manner, thereby minimizing the possibility of insertional mutagenesis and variability of inserted gene expression characteristic of retroviral infection. In addition, wild-type adeno-associated virus infections have been followed in tissue culture for greater than 100 passages in the absence of selective pressure, implying that the adeno-associated virus genomic integration is a relatively stable event. The adeno-associated virus can also function in an extrachromosomal fashion.

Other vectors include plasmid vectors. Plasmid vectors have been extensively described in the art and are well-known to those of skill in the art. See e.g., Sambrook et al., "Molecular Cloning: A Laboratory Manual," Second Edition, Cold Spring Harbor Laboratory Press, 1989. In the last few years, plasmid vectors have been found to be particularly advantageous for delivering genes to cells *in vivo* because of their inability to replicate within and integrate into a host genome. These plasmids, however, having a promoter compatible with the host cell, can express a peptide from a gene operatively encoded within the plasmid. Some commonly used plasmids include pBR322, pUC18, pUC19, pRC/CMV, SV40, and pBlueScript. Other plasmids are well-known to those of ordinary skill in the art. Additionally, plasmids may be custom designed using restriction enzymes and ligation reactions to remove and add specific fragments of DNA.

It has recently been discovered that gene carrying plasmids can be delivered to the immune system using bacteria. Modified forms of bacteria such as *Salmonella* can be transfected with the plasmid and used as delivery vehicles. The bacterial delivery vehicles can be administered to a host subject orally or by other administration means. The bacteria deliver the plasmid to immune cells, e.g. B cells, dendritic cells, likely by passing through the gut barrier. High levels of immune protection have been established using this methodology. Such methods of delivery are useful for the aspects of the invention utilizing systemic delivery of antigen, S-chiral CpG oligonucleotide and/or other therapeutic agent.

S-chiral CpG oligonucleotide can be combined with other therapeutic agents such as adjuvants to enhance immune responses. The CpG oligonucleotide and other therapeutic agent may be administered simultaneously or sequentially. When the other therapeutic agents are administered simultaneously they can be administered in the same or separate formulations, but are administered at the same time. The other therapeutic agents are administered sequentially with one another and with CpG, when the administration of the other therapeutic agents and the CpG is temporally separated. The separation in time between the administration of these

compounds may be a matter of minutes or it may be longer. Other therapeutic agents include but are not limited to adjuvants, cytokines, antibodies, antigens, etc.

The S-chiral CpG oligonucleotide is useful as an adjuvant for inducing a systemic immune response. Thus it can be delivered to a subject exposed to an antigen to produce an enhanced immune response to the antigen.

In addition to the S-chiral CpG oligonucleotide, the compositions of the invention may also be administered with non-nucleic acid adjuvants. A "non-nucleic acid adjuvant" is any molecule or compound except for the CpG oligonucleotides described herein which can stimulate the humoral and/or cellular immune response. Non-nucleic acid adjuvants include, for instance, adjuvants that create a depo effect, immune stimulating adjuvants, and adjuvants that create a depo effect and stimulate the immune system.

An "adjuvant that creates a depo effect" as used herein is an adjuvant that causes the antigen to be slowly released in the body, thus prolonging the exposure of immune cells to the antigen. This class of adjuvants includes but is not limited to alum (e.g., aluminum hydroxide, aluminum phosphate); or emulsion-based formulations including mineral oil, non-mineral oil, water-in-oil or oil-in-water-in oil emulsion, oil-in-water emulsions such as Seppic ISA series of Montanide adjuvants (e.g., Montanide ISA 720, AirLiquide, Paris, France); MF-59 (a squalene-in-water emulsion stabilized with Span 85 and Tween 80; Chiron Corporation, Emeryville, CA; and PROVAX (an oil-in-water emulsion containing a stabilizing detergent and a micelle-forming agent; IDEC, Pharmaceuticals Corporation, San Diego, CA).

An "immune stimulating adjuvant" is an adjuvant that causes activation of a cell of the immune system. It may, for instance, cause an immune cell to produce and secrete cytokines. This class of adjuvants includes but is not limited to saponins purified from the bark of the *Q. saponaria* tree, such as QS21 (a glycolipid that elutes in the 21st peak with HPLC fractionation; Aquila Biopharmaceuticals, Inc., Worcester, MA); poly[di(carboxylatophenoxy)phosphazene (PCPP polymer; Virus Research Institute, USA); derivatives of lipopolysaccharides such as monophosphoryl lipid A (MPL; Ribi ImmunoChem Research, Inc., Hamilton, MT), muramyl dipeptide (MDP; Ribi) and threonyl-muramyl dipeptide (t-MDP; Ribi); OM-174 (a glucosamine disaccharide related to lipid A; OM Pharma SA, Meyrin, Switzerland); and Leishmania elongation factor (a purified *Leishmania* protein; Corixa Corporation, Seattle, WA).

"Adjuvants that create a depo effect and stimulate the immune system" are those compounds which have both of the above-identified functions. This class of adjuvants includes

but is not limited to ISCOMS (Immunostimulating complexes which contain mixed saponins, lipids and form virus-sized particles with pores that can hold antigen; CSL, Melbourne, Australia); SB-AS2 (SmithKline Beecham adjuvant system #2 which is an oil-in-water emulsion containing MPL and QS21; SmithKline Beecham Biologicals [SBB], Rixensart, Belgium); SB-AS4 (SmithKline Beecham adjuvant system #4 which contains alum and MPL; SBB, Belgium); non-ionic block copolymers that form micelles such as CRL 1005 (these contain a linear chain of hydrophobic polyoxpropylene flanked by chains of polyoxyethylene; Vaxcel, Inc., Norcross, GA); and Syntex Adjuvant Formulation (SAF, an oil-in-water emulsion containing Tween 80 and a nonionic block copolymer; Syntex Chemicals, Inc., Boulder, CO).

10 The S-chiral CpG oligonucleotide is also useful as a mucosal adjuvant. It has previously been discovered that both systemic and mucosal immunity are induced by mucosal delivery of CpG oligonucleotides. The systemic immunity induced in response to CpG oligonucleotides included both humoral and cell-mediated responses to specific antigens that were not capable of inducing systemic immunity when administered alone to the mucosa. Furthermore, both CpG
15 oligonucleotides and cholera toxin (CT, a mucosal adjuvant that induces a Th2-like response) induced CTL. This was surprising since with systemic immunization, the presence of Th2-like antibodies is normally associated with a lack of CTL (Schirmbeck *et al.*, 1995).

Additionally, CpG oligonucleotides were found to induce a mucosal response at both local (e.g., lung) and remote (e.g., lower digestive tract) mucosal sites. Although CpG
20 oligonucleotide was similar to CT for induction of systemic antibodies (IgG) and local mucosal antibodies (IgA), significant levels of IgA antibodies were induced at a distant mucosal site only by CpG oligonucleotide and not by CT. This was surprising because CT is generally considered to be a highly effective mucosal adjuvant. Another manner in which CpG oligonucleotide was superior to CT was with respect to the Th-type of response. As has been previously reported
25 (Snider 1995), CT induces predominantly IgG1 isotype of antibodies, which are indicative of Th2-type response. In contrast, CpG oligonucleotide was more Th1 with predominantly IgG2a antibodies, especially after boost or when the two adjuvants were combined. Th1-type antibodies in general have better neutralizing capabilities, and furthermore, a Th2 response in the lung is highly undesirable because it is associated with asthma (Kay, 1996, Hogg, 1997). Thus
30 the use of CpG oligonucleotide as a mucosal adjuvant has benefits that other mucosal adjuvants cannot achieve. The S-chiral CpG oligonucleotides of the invention are useful as mucosal adjuvants for induction of both a systemic and a mucosal immune response.

Mucosal adjuvants referred to as non-oligonucleotide mucosal adjuvants may also be administered with the S-chiral CpG oligonucleotide. A "non-oligonucleotide mucosal adjuvant" as used herein is an adjuvant other than a S-chiral CpG oligonucleotide that is capable of inducing a mucosal immune response in a subject when administered to a mucosal surface in conjunction with an antigen. Mucosal adjuvants include but are not limited to Bacterial toxins: e.g., Cholera toxin (CT), CT derivatives including but not limited to CT B subunit (CTB) (Wu et al., 1998, Tochikubo et al., 1998); CTD53 (Val to Asp) (Fontana et al., 1995); CTK97 (Val to Lys) (Fontana et al., 1995); CTK104 (Tyr to Lys) (Fontana et al., 1995); CTD53/K63 (Val to Asp, Ser to Lys) (Fontana et al., 1995); CTH54 (Arg to His) (Fontana et al., 1995); CTN107 (His to Asn) (Fontana et al., 1995); CTE114 (Ser to Glu) (Fontana et al., 1995); CTE112K (Glu to Lys) (Yamamoto et al., 1997a); CTS61F (Ser to Phe) (Yamamoto et al., 1997a, 1997b); CTS106 (Pro to Lys) (Douce et al., 1997, Fontana et al., 1995); and CTK63 (Ser to Lys) (Douce et al., 1997, Fontana et al., 1995), Zonula occludens toxin, zot, Escherichia coli heat-labile enterotoxin, Labile Toxin (LT), LT derivatives including but not limited to LT B subunit (LTB) (Verweij et al., 1998); LT7K (Arg to Lys) (Komase et al., 1998, Douce et al., 1995); LT61F (Ser to Phe) (Komase et al., 1998); LT112K (Glu to Lys) (Komase et al., 1998); LT118E (Gly to Glu) (Komase et al., 1998); LT146E (Arg to Glu) (Komase et al., 1998); LT192G (Arg to Gly) (Komase et al., 1998); LTK63 (Ser to Lys) (Marchetti et al., 1998, Douce et al., 1997, 1998, Di Tommaso et al., 1996); and LTR72 (Ala to Arg) (Giuliani et al., 1998), Pertussis toxin, PT. (Lycke et al., 1992, Spangler BD, 1992, Freytag and Clemments, 1999, Roberts et al., 1995, Wilson et al., 1995) including PT-9K/129G (Roberts et al., 1995, Cropley et al., 1995); Toxin derivatives (see below) (Holmgren et al., 1993, Verweij et al., 1998, Rappuoli et al., 1995, Freytag and Clements, 1999); Lipid A derivatives (e.g., monophosphoryl lipid A, MPL) (Sasaki et al., 1998, Vancott et al., 1998; Muramyl Dipeptide (MDP) derivatives (Fukushima et al., 1996, Ogawa et al., 1989, Michalek et al., 1983, Morisaki et al., 1983); Bacterial outer membrane proteins (e.g., outer surface protein A (OspA) lipoprotein of *Borrelia burgdorferi*, outer membrane protine of *Neisseria meningitidis*) (Marinaro et al., 1999, Van de Verg et al., 1996); Oil-in-water emulsions (e.g., MF59) (Barchfield et al., 1999, Verschoor et al., 1999, O'Hagan, 1998); Aluminum salts (Isaka et al., 1998, 1999); and Saponins (e.g., QS21) Aquila Biopharmaceuticals, Inc., Worster, MA) (Sasaki et al., 1998, MacNeal et al., 1998), ISCOMS, MF-59 (a squalene-in-water emulsion stabilized with Span 85 and Tween 80; Chiron Corporation, Emeryville, CA); the Seppic ISA series of Montanide adjuvants (e.g., Montanide

ISA 720; AirLiquide, Paris, France); PROVAX (an oil-in-water emulsion containing a stabilizing detergent and a micell-forming agent; IDEC Pharmaceuticals Corporation, San Diego, CA); Syntex Adjuvant Formulation (SAF; Syntex Chemicals, Inc., Boulder, CO); poly[di(carboxylatophenoxy)phosphazene (PCPP polymer; Virus Research Institute, USA) and
5 Leishmania elongation factor (Corixa Corporation, Seattle, WA).

Immune responses can also be induced or augmented by the co-administration or co-linear expression of cytokines (Bueler & Mulligan, 1996; Chow *et al.*, 1997; Geissler *et al.*, 1997; Iwasaki *et al.*, 1997; Kim *et al.*, 1997) or B-7 co-stimulatory molecules (Iwasaki *et al.*, 1997; Tsuji *et al.*, 1997) with the chiral CpG oligonucleotides. The cytokines can be administered directly
10 with CpG oligonucleotides or may be administered in the form of a nucleic acid vector that encodes the cytokine, such that the cytokine can be expressed *in vivo*. In one embodiment, the cytokine is administered in the form of a plasmid expression vector. The term "cytokine" is used as a generic name for a diverse group of soluble proteins and peptides which act as humoral regulators at nano- to picomolar concentrations and which, either under normal or pathological
15 conditions, modulate the functional activities of individual cells and tissues. These proteins also mediate interactions between cells directly and regulate processes taking place in the extracellular environment. Examples of cytokines include, but are not limited to IL-1, IL-2, IL-4, IL-5, IL-6, IL-7, IL-10, IL-12, IL-15, IL-18, granulocyte-macrophage colony stimulating factor (GM-CSF), granulocyte colony stimulating factor (GCSF), interferon- γ (γ -IFN), IFN- α , tumor
20 necrosis factor (TNF), TGF- β , FLT-3 ligand, and CD40 ligand.

Cytokines play a role in directing the T cell response. Helper (CD4+) T cells orchestrate the immune response of mammals through production of soluble factors that act on other immune system cells, including other T cells. Most mature CD4+ T helper cells express one of two cytokine profiles: Th1 or Th2. Th1 cells express IL-3, IL-4, IL-5, IL-6, IL-9, IL-10, IL-13,
25 GM-CSF and low levels of TNF- α . The TH1 subset promotes delayed-type hypersensitivity, cell-mediated immunity, and immunoglobulin class switching to IgG_{2a}. The Th2 subset induces humoral immunity by activating B cells, promoting antibody production, and inducing class switching to IgG₁ and IgE. In some embodiments it is preferred that the cytokine be a Th1 cytokine.

30 The S-chiral oligonucleotides are also useful for redirecting an immune response from a Th2 immune response to a Th1 immune response. Redirection of an immune response from a Th2 to a Th1 immune response can be assessed by measuring the levels of cytokines produced in

response to the oligonucleotide (*e.g.*, by inducing monocytic cells and other cells to produce Th1 cytokines, including IL-12, IFN- γ and GM-CSF). The redirection of the immune response from a Th2 to a Th1 response is particularly useful for the treatment or prevention of asthma. For instance, an "effective amount" for treating asthma can be that amount; useful for redirecting a

5 Th2 type of immune response that is associated with asthma to a Th1 type of response. Th2 cytokines, especially IL-4 and IL-5 are elevated in the airways of asthmatic subjects. These cytokines promote important aspects of the asthmatic inflammatory response, including IgE isotype switching, eosinophil chemotaxis and activation and mast cell growth. Th1 cytokines, especially IFN- γ and IL-12, can suppress the formation of Th2 clones and production of Th2

10 cytokines.

The S-chiral oligonucleotides are also useful for improving survival, differentiation, activation and maturation of dendritic cells. It has previously been shown that CpG has the unique capability to promote cell survival, differentiation, activation and maturation of dendritic cells. Dendritic precursor cells isolated from blood by immunomagnetic cell sorting develop

15 morphologic and functional characteristics of dendritic cells during a two day incubation with GM-CSF. Without GM-CSF these cells undergo apoptosis. It was previously found that CpG was superior to GM-CSF in promoting survival and differentiation of dendritic cells (MHC II expression, cell size, granularity). CpG was also found to induce maturation of dendritic cells. Since dendritic cells form the link between the innate and the acquired immune system, by

20 presenting antigens as well as through their expression of pattern recognition receptors which detect microbial molecules like LPS in their local environment, the ability to activate dendritic cells with CpG supports the use of CpG-based strategies for *in vivo* and *ex-vivo* immunotherapy against disorders such as cancer and allergic or infectious diseases. The S-chiral CpG oligonucleotides are also useful for activating and inducing maturation of dendritic cells.

25 CpG nucleic acids also increase natural killer cell lytic activity and antibody dependent cellular cytotoxicity (ADCC). ADCC can be performed using a S-chiral CpG oligonucleotide in combination with an antibody specific for a cellular target, such as a cancer cell. When the S-chiral CpG oligonucleotide is administered to a subject in conjunction with the antibody the subjects immune system is induced to kill the tumor cell. The antibodies useful in the ADCC

30 procedure include antibodies which interact with a cell in the body. Many such antibodies specific for cellular targets have been described in the art and many are commercially available. These antibodies include but are not limited to those presented in the Table below.

Antibody-Based Immune Therapy Product Development (by companies)

	Antibody Classification	Indication	Drug Name/Antibody	Company(ies)
5	1	non-Hodgkin's lymphoma	Rituxan™ (rituximab, Mabthera) (IDEC-C2B8, chimeric murine/human anti-CD20 MAb)	IDEC/Genentech, Inc./Hoffmann-La Roche (first monoclonal antibody licensed for the treatment of cancer in the U.S.)
	1	Adjuvant therapy for colorectal (Dukes-C)	Panorex® (17-1A) (murine monoclonal antibody)	Centocor/Glaxo/Ajinomoto
	1	Pancreatic, lung, breast, ovary	Panorex® (17-1A) (chimeric murine monoclonal antibody)	Centocor/Ajinomoto
	1	non-small cell lung, prostate (adjuvant)	3622W94 MAb that binds to EGP40 (17-1A) pancarcinoma antigen on adenocarcinomas	Glaxo Wellcome plc
	2	Breast/ovarian	Herceptin, anti-Her2 hMAb	Genentech/Hoffmann-La Roche
10	2	Renal cell	C225 (chimeric monoclonal antibody to epidermal growth factor receptor (EGFr))	ImClone Systems
	2	Breast	C225 (chimeric anti-EGFr monoclonal antibody) + taxol	ImClone Systems
	2	prostate	C225 (chimeric anti-EGFr monoclonal antibody) + doxorubicin	ImClone Systems (licensed from RPR)
	2	prostate	C225 (chimeric anti-EGFr monoclonal antibody) + adriamycin	ImClone Systems
	3	Small cell lung	BEC2 (anti-idiotypic MAb, mimics the GD, epitope) (with BCG7)	ImClone Systems
15	3	?	Overax (B43.13, anti-idiotypic CA125, mouse MAb)	Altarex, Canada
	3	Melanoma	BEC2 (anti-idiotypic MAb, mimics the GD, epitope)	ImClone Systems
	3	Melanoma, small-cell lung	4B5 anti-idiotypic Ab	Novopharm Biotech, Inc.
	4	Lung, breast, prostate, colorectal	Anti-VEGF, RhuMAb (inhibits angiogenesis)	Genentech
	5	Breast, ovarian	MDX-210 (humanized anti-HER-2 bispecific antibody)	Medarex/Novartis
20	5	Prostate, non-small cell lung, pancreatic, breast	MDX-210 (humanized anti-HER-2 bispecific antibody)	Medarex/Novartis
	5	Renal and colon	MDX-210 (humanized anti-HER-2 bispecific antibody)	Medarex/Novartis
	5	Acute myeloid leukemia	MDX-22 (humanized bispecific antibody, MAb-conjugates) (complement cascade activators)	Medarex
	5	Cancer	MDX-210 (humanized anti-HER-2 bispecific antibody)	Medarex
	5	Lung, colon, prostate, ovarian, endometrial, pancreatic and gastric	MDX-220 (bispecific for tumors that express TAG-72)	Medarex
25	5	Prostate	MDX-210 (humanized anti-HER-2 bispecific antibody)	Medarex/Novartis
	5	EGF receptor cancers (head & neck, prostate, lung, bladder, cervical, ovarian)	MDX-447 (humanized anti-EGF receptor bispecific antibody)	Medarex/Merck KGaA
	5	Comb. Therapy with G-CSF for various cancers, esp. breast	MDX-210 (humanized anti-HER-2 bispecific antibody)	Medarex/Novartis

5	Melanoma, glioma, neuroblastoma	MDX-260 bispecific, targets GD-2	Medarex, Inc.
	Bone metastases	Quadramet (CYT-424) radiotherapeutic agent	Cytogen Corp.
	non-Hodgkin's lymphoma	IDEC-Y2B8 (murine, anti-CD20 MAb labeled with Yttrium-90)	IDEC
	non-Hodgkin's lymphoma	Oncolym (Lym-1 monoclonal antibody linked to 131 iodine)	Techniclone International/Alpha Therapeutics
	Acute myeloid leukemia	SMART M195 Ab, humanized	Protein Design Labs
10	non-Hodgkin's lymphoma	¹²⁵ I LYM-1 (Oncolym TM)	Techniclone Corporation/Cambridge Antibody Technology
	Acute promyelocytic leukemia	ATRAGEN®	Aronex Pharmaceuticals, Inc.
	Head & neck, non-small cell lung cancer	C225 (chimeric anti-EGFr monoclonal antibody) + cisplatin or radiation	ImClone Systems
	non-Hodgkin's lymphoma	Bexxar (anti-CD20 Mab labeled with ¹²⁵ I)	Coulter Pharma (Clinical results have been positive, but the drug has been associated with significant bone marrow toxicity)
	Kaposi's sarcoma	ATRAGEN®	Aronex Pharmaceuticals, Inc.
15	B cell lymphoma	Rituxan TM (MAb against CD20) pan-B Ab in combo. with chemotherapy	IDEC Pharmaceuticals Corp./Genentech
	Chronic lymphocytic leukemia (CLL)	LDP-03, huMAb to the leukocyte antigen CAMPATH	LeukoSite/Ilex Oncology
	Cancer	ior t6 (anti CD6, murine MAb) CTCL	Center of Molecular Immunology
	Acute myelogenous leukemia (AML)	MDX-11 (complement activating receptor (CAR) monoclonal antibody)	Medarex
	Ex vivo bone marrow purging in acute myelogenous leukemia (AML)	MDX-11 (complement activating receptor (CAR) monoclonal antibody)	Medarex
20	Ovarian	OV103 (Yttrium-90 labelled antibody)	Cytogen
	Prostate	OV103 (Yttrium-90 labelled antibody)	Cytogen
	non-Hodgkin's lymphoma	ATRAGEN®	Aronex Pharmaceuticals, Inc.
	Leukemia, lymphoma	Zenapax (SMART Anti-Tac (IL-2 receptor) Ab, humanized)	Protein Design Labs
	Acute promyelocytic leukemia	SMART M195 Ab, humanized	Protein Design Labs
25	Melanoma	MELIMMUNE-2 (murine monoclonal antibody therapeutic vaccine)	IDEC
	Melanoma	MELIMMUNE-1 (murine monoclonal antibody therapeutic vaccine)	IDEC
	Colorectal and other	CEACIDE TM (I-131)	Immunomedics, Inc.
	non-Hodgkin's B cell lymphoma	Pretarget TM radioactive antibodies	NeoRx
	Cancer	NovoMAB-G2 (pancarcinoma specific Ab)	Novopharm Biotech, Inc.
30	Brain	TNT (chimeric MAb to histone antigens)	Techniclone Corporation/Cambridge Antibody Technology
	Brain	TNT (chimeric MAb to histone antigens)	Techniclone International/Cambridge Antibody Technology
	Brain, melanomas, neuroblastomas	Gliomab-H (Monoclonals - Humanized Abs)	Novopharm
	Colorectal	GNI-250 MAb	Genetics Institute/AHP
	Cancer	EMD-72000 (chimeric-EGF antagonist)	Merck KgaA
	non-Hodgkin's B-cell lymphoma	LymphoCide (humanized LL2 antibody)	Immunomedics
	Acute myelogenous leukemia	CMA 676 (monoclonal antibody conjugate)	Immunex/AHP
	Colon, lung, pancreatic	Monopharm-C	Novopharm Biotech, Inc.

	Radioimmunotherapy	for eg/r3 (anti EGF-R humanized Ab)	Center of Molecular Immunology
	Colorectal	for c5 (murine MAb colorectal) for radioimmunotherapy	Center of Molecular Immunology
	Breast cancer	BABS (biosynthetic antibody binding site) proteins	Creative BioMolecules/Chiron
	Tumor-associated angiogenesis	FLK-2 (monoclonal antibody to fetal liver kinase-2 (FLK-2))	ImClone Systems/Chugai
5	Small-cell lung	Humanized MAb/small-drug conjugate	ImmunoGen, Inc.
	Cancer	ANA Ab	Procyon Biopharma, Inc.
	B-cell lymphoma	SMART 1D10 Ab	Protein Design Labs
	Breast, lung, colon	SMART ABL 364 Ab	Protein Design Labs/Novartis
10	Colorectal	ImmuRAIT-CEA	Immunomedics, Inc.

The invention also includes a method for inducing antigen non-specific innate immune activation and broad spectrum resistance to infectious challenge. The term "antigen non-specific innate immune activation" as used herein refers to the activation of immune cells other than B cells and for instance can include the activation of NK cells, T cells or other immune cells that can respond in an antigen independent fashion or some combination of these cells. A broad spectrum resistance to infectious challenge is induced because the immune cells are in active form and are primed to respond to any invading compound or microorganism. The cells do not have to be specifically primed against a particular antigen. This is particularly useful in biowarfare, and the other circumstances described above such as travelers.

The invention also is based on the finding that the R stereoisomer of CpG containing nucleic acids reduces an immune response. A CpG nucleic acid composed partially or completely of the R stereoisomer may be used to prevent an immune response when it is desirable to do so. As used herein the term "prevent" refers to inhibiting the development of an immune response, as well as to decreasing the rate of a developing immune response.

The R stereoisomer is also useful for preventing an immune response caused by administration of an immunostimulatory compound such as an immunostimulatory CpG compound. For instance, when the immunostimulatory CpG nucleic acid has been administered in too high of a dose and causes more of an immune response than is desirable the R stereoisomer may be administered to antagonize the immune stimulating effect of CpG. Alternatively if only a transient immune stimulating effect is desired the R stereoisomer can be administered to reduce the immune stimulation. Thus it is possible to regulate the timing of a CpG induced immune response by administering R stereoisomer at the appropriate time. Thus, the R stereoisomer can be used as a therapeutic to treat a subject having an excessive immune

response. An "excessive immune response" as used herein refers to an immune response which is greater than that response which is desirable. For instance an excessive immune response may be associated with harmful side effects. An excessive immune response can be induced in a subject who has received an immune stimulating compound, such as an immunostimulatory CpG
5 oligonucleotide.

The R stereoisomer of CpG is also useful for the therapeutic treatment of disorders mediated by an immune response. Thus the R stereoisomer can be used as a therapeutic to treat a subject having or at risk of having an inflammatory disease. An "inflammatory disease or condition" as used herein refers to any condition characterized by local inflammation at a site of
10 injury or infection and includes but is not limited to autoimmune diseases, certain forms of infectious inflammatory states, such as inflammatory bowel disease, psoriasis, gingivitis, systemic lupus erythematosus, sepsis, meningitis, cerebral edema, arthritis, nephritis, adult respiratory distress syndrome, pancreatitis, myositis, neuritis, connective tissue diseases, phlebitis, arteritis, vasculitis, allergy, anaphylaxis, ehrlichiosis, gout, organ transplants and/or
15 ulcerative colitis and virtually any other condition characterized by unwanted immune cell activation.

The stimulation index of a particular immunostimulatory S-chiral CpG oligonucleotide can be tested in various immune cell assays. Preferably, the stimulation index of the CpG oligonucleotide with regard to B cell proliferation is at least about 5, preferably at least about 10,
20 more preferably at least about 15 and most preferably at least about 20 as determined by incorporation of ^3H uridine in a murine B cell culture, which has been contacted with 20 μM of oligonucleotide for 20h at 37°C and has been pulsed with 1 μCi of ^3H uridine; and harvested and counted 4h later as described in detail in PCT Published Patent Applications PCT/US95/01570 and PCT/US97/19791 claiming priority to U.S. Serial Nos. 08/386,063 and 08/960,774, filed on
25 February 7, 1995 and October 30, 1997 respectively. For use *in vivo*, for example, it is important that the S-chiral CpG oligonucleotide be capable of effectively inducing an immune response, such as antibody production. The inhibitory index of a R-chiral CpG oligonucleotide can be assessed in the same types of assays except that inhibition is measured rather than stimulation.

Nucleic acids containing an appropriate unmethylated CpG can be effective in any
30 vertebrate. Different nucleic acids containing an unmethylated CpG can cause optimal immune stimulation depending on the mammalian species. Thus an oligonucleotide causing optimal

stimulation or inhibition in humans may not cause optimal stimulation or inhibition in a mouse, and vice versa. One of skill in the art can identify the optimal oligonucleotides useful for a particular mammalian species of interest using routine assays described herein and/or known in the art, using the guidance supplied herein.

- 5 The chiral CpG oligonucleotide may be directly administered to the subject or may be administered in conjunction with a nucleic acid delivery complex. A "nucleic acid delivery complex" shall mean a nucleic acid molecule associated with (e.g. ionically or covalently bound to; or encapsulated within) a targeting means (e.g. a molecule that results in higher affinity binding to target cell (e.g. B cell surfaces and/or increased cellular uptake by target cells).
- 10 Examples of nucleic acid delivery complexes include nucleic acids associated with: a sterol (e.g. cholesterol), a lipid (e.g. a cationic lipid, virosome or liposome), or a target cell specific binding agent (e.g. a ligand recognized by target cell specific receptor). Preferred complexes may be sufficiently stable *in vivo* to prevent significant uncoupling prior to internalization by the target cell. However, the complex can be cleavable under appropriate conditions within the cell so that
- 15 the nucleic acid is released in a functional form.

- Delivery vehicles for delivering antigen to surfaces have been described. The CpG oligonucleotide and/or the antigen and/or other therapeutics may be administered alone (e.g. in saline or buffer) or using any delivery vehicles known in the art. For instance the following delivery vehicles have been described: Cochleates (Gould-Fogerite et al., 1994, 1996);
- 20 Emulsomes (Vancott et al., 1998, Lowell et al., 1997); ISCOMs (Mowat et al., 1993, Carlsson et al., 1991, Hu et al., 1998, Morein et al., 1999); Liposomes (Childers et al., 1999, Michalek et al., 1989, 1992, de Haan 1995a, 1995b); Live bacterial vectors (e.g., *Salmonella*, *Escherichia coli*, *Bacillus calmatte-guerin*, *Shigella*, *Lactobacillus*) (Hone et al., 1996, Pouwels et al., 1998, Chatfield et al., 1993, Stover et al., 1991, Nugent et al., 1998); Live viral vectors (e.g., Vaccinia,
- 25 adenovirus, Herpes Simplex) (Gallichan et al., 1993, 1995, Moss et al., 1996, Nugent et al., 1998, Flexner et al., 1988, Morrow et al., 1999); Microspheres (Gupta et al., 1998, Jones et al., 1996, Maloy et al., 1994, Moore et al., 1995, O'Hagan et al., 1994, Eldridge et al., 1989); Nucleic acid vaccines (Fynan et al., 1993, Kuklin et al., 1997, Sasaki et al., 1998, Okada et al., 1997, Ishii et al., 1997); Polymers (e.g. carboxymethylcellulose, chitosan) (Hamajima et al.,
- 30 1998, Jabbal-Gill et al., 1998); Polymer rings (Wyatt et al., 1998); Proteosomes (Vancott et al., 1998, Lowell et al., 1988, 1996, 1997); Sodium Fluoride (Hashi et al., 1998); Transgenic plants

(Tacket et al., 1998, Mason et al., 1998, Haq et al., 1995); Virosomes (Gluck et al., 1992, Mengiardi et al., 1995, Cryz et al., 1998); Virus-like particles (Jiang et al., 1999, Leibl et al., 1998). Other delivery vehicles are known in the art and some additional examples are provided below in the discussion of vectors.

5 The term "effective amount" of a CpG oligonucleotide refers to the amount necessary or sufficient to realize a desired biologic effect. For example, an effective amount of an S-chiral CpG oligonucleotide for inducing mucosal immunity is that amount necessary to cause the development of IgA in response to an antigen upon exposure to the antigen, whereas that amount required for inducing systemic immunity is that amount necessary to cause the development of
10 IgG in response to an antigen upon exposure to the antigen. Combined with the teachings provided herein, by choosing among the various active compounds and weighing factors such as potency, relative bioavailability, patient body weight, severity of adverse side-effects and preferred mode of administration, an effective prophylactic or therapeutic treatment regimen can be planned which does not cause substantial toxicity and yet is entirely effective to treat the
15 particular subject. The effective amount for any particular application can vary depending on such factors as the disease or condition being treated, the particular CpG oligonucleotide being administered (e.g. the number of unmethylated CpG motifs or their location in the nucleic acid, the degree of chirality to the oligonucleotide), the antigen, the size of the subject, or the severity of the disease or condition. One of ordinary skill in the art can empirically determine the
20 effective amount of a particular CpG oligonucleotide and/or antigen and/or other therapeutic agent without necessitating undue experimentation.

Subject doses of the compounds described herein typically range from about 80 mg/day to 16,000 mg/day, more typically from about 800 mg/day to 8000 mg/day, and most typically from about 800 mg/day to 4000 mg/day. Stated in terms of subject body weight, typical dosages
25 range from about 1 to 200 mg/kg/day, more typically from about 10 to 100 mg/kg/day, and most typically from about 10 to 50 mg/kg/day. Stated in terms of subject body surface areas, typical dosages range from about 40 to 8000 mg/m²/day, more typically from about 400 to 4000 mg/m²/day, and most typically from about 400 to 2000 mg/m²/day.

In some embodiments, particularly when the CpG is in a plasmid vector, at least 50 mg of
30 the CpG is administered to a subject. In other embodiments at least 75 mg, 100 mg, 200 mg, 300 mg, 400 mg, 500 mg and every integer in between of the CpG is administered to the subject.

For any compound described herein the therapeutically effective amount can be initially determined from cell culture assays. For instance the effective amount of S-chiral CpG oligonucleotide useful for inducing B cell activation can be assessed using the in vitro assays described above with respect to stimulation index. The stimulation index can be used to
5 determine an effective amount of the particular oligonucleotide for the particular subject, and the dosage can be adjusted upwards or downwards to achieve the desired levels in the subject. Therapeutically effective amounts can also be determined from animal models. A therapeutically effective dose can also be determined from human data for CpG oligonucleotides which have been tested in humans (human clinical trials have been initiated) and for compounds
10 which are known to exhibit similar pharmacological activities, such as other adjuvants, e.g., LT and other antigens for vaccination purposes. The applied dose can be adjusted based on the relative bioavailability and potency of the administered compound. Adjusting the dose to achieve maximal efficacy based on the methods described above and other methods as are well-known in the art is well within the capabilities of the ordinarily skilled artisan.

15 The formulations of the invention are administered in pharmaceutically acceptable solutions, which may routinely contain pharmaceutically acceptable concentrations of salt, buffering agents, preservatives, compatible carriers, adjuvants, and optionally other therapeutic ingredients.

For use in therapy, an effective amount of the CpG oligonucleotide can be administered
20 to a subject by any mode that delivers the oligonucleotide to the desired surface, e.g., mucosal, systemic. "Administering" the pharmaceutical composition of the present invention may be accomplished by any means known to the skilled artisan. Preferred routes of administration include but are not limited to oral, parenteral, intramuscular, intranasal, intratracheal, inhalation, ocular, vaginal, and rectal.

25 For oral administration, the compounds (i.e., CpG oligonucleotides, antigen, other therapeutic agent) can be formulated readily by combining the active compound(s) with pharmaceutically acceptable carriers well known in the art. Such carriers enable the compounds of the invention to be formulated as tablets, pills, dragees, capsules, liquids, gels, syrups, slurries, suspensions and the like, for oral ingestion by a subject to be treated. Pharmaceutical
30 preparations for oral use can be obtained as solid excipient, optionally grinding a resulting mixture, and processing the mixture of granules, after adding suitable auxiliaries, if desired, to

obtain tablets or dragee cores. Suitable excipients are, in particular, fillers such as sugars, including lactose, sucrose, mannitol, or sorbitol; cellulose preparations such as, for example, maize starch, wheat starch, rice starch, potato starch, gelatin, gum tragacanth, methyl cellulose, hydroxypropylmethyl-cellulose, sodium carboxymethylcellulose, and/or polyvinylpyrrolidone (PVP). If desired, disintegrating agents may be added, such as the cross-linked polyvinyl pyrrolidone, agar, or alginic acid or a salt thereof such as sodium alginate. Optionally the oral formulations may also be formulated in saline or buffers for neutralizing internal acid conditions or may be administered without any carriers.

Dragee cores are provided with suitable coatings. For this purpose, concentrated sugar solutions may be used, which may optionally contain gum arabic, talc, polyvinyl pyrrolidone, carbopol gel, polyethylene glycol, and/or titanium dioxide, lacquer solutions, and suitable organic solvents or solvent mixtures. Dyestuffs or pigments may be added to the tablets or dragee coatings for identification or to characterize different combinations of active compound doses.

Pharmaceutical preparations which can be used orally include push-fit capsules made of gelatin, as well as soft, sealed capsules made of gelatin and a plasticizer, such as glycerol or sorbitol. The push-fit capsules can contain the active ingredients in admixture with filler such as lactose, binders such as starches, and/or lubricants such as talc or magnesium stearate and, optionally, stabilizers. In soft capsules, the active compounds may be dissolved or suspended in suitable liquids, such as fatty oils, liquid paraffin, or liquid polyethylene glycols. In addition, stabilizers may be added. Microspheres formulated for oral administration may also be used. Such microspheres have been well defined in the art. All formulations for oral administration should be in dosages suitable for such administration.

For buccal administration, the compositions may take the form of tablets or lozenges formulated in conventional manner.

For administration by inhalation, the compounds for use according to the present invention may be conveniently delivered in the form of an aerosol spray presentation from pressurized packs or a nebulizer, with the use of a suitable propellant, *e.g.*, dichlorodifluoromethane, trichlorofluoromethane, dichlorotetrafluoroethane, carbon dioxide or other suitable gas. In the case of a pressurized aerosol the dosage unit may be determined by

providing a valve to deliver a metered amount. Capsules and cartridges of *e.g.* gelatin for use in an inhaler or insufflator may be formulated containing a powder mix of the compound and a suitable powder base such as lactose or starch.

The compounds, when it is desirable to deliver them systemically, may be formulated for
5 parenteral administration by injection, *e.g.*, by bolus injection or continuous infusion.

Formulations for injection may be presented in unit dosage form, *e.g.*, in ampoules or in multi-dose containers, with an added preservative. The compositions may take such forms as suspensions, solutions or emulsions in oily or aqueous vehicles, and may contain formulatory agents such as suspending, stabilizing and/or dispersing agents.

10 Pharmaceutical formulations for parenteral administration include aqueous solutions of the active compounds in water-soluble form. Additionally, suspensions of the active compounds may be prepared as appropriate oily injection suspensions. Suitable lipophilic solvents or vehicles include fatty oils such as sesame oil, or synthetic fatty acid esters, such as ethyl oleate or triglycerides, or liposomes. Aqueous injection suspensions may contain substances which
15 increase the viscosity of the suspension, such as sodium carboxymethyl cellulose, sorbitol, or dextran. Optionally, the suspension may also contain suitable stabilizers or agents which increase the solubility of the compounds to allow for the preparation of highly concentrated solutions.

Alternatively, the active compounds may be in powder form for constitution with a
20 suitable vehicle, *e.g.*, sterile pyrogen-free water, before use.

The compounds may also be formulated in rectal or vaginal compositions such as suppositories or retention enemas, *e.g.*, containing conventional suppository bases such as cocoa butter or other glycerides.

In addition to the formulations described previously, the compounds may also be
25 formulated as a depot preparation. Such long acting formulations may be formulated with suitable polymeric or hydrophobic materials (for example as an emulsion in an acceptable oil) or ion exchange resins, or as sparingly soluble derivatives, for example, as a sparingly soluble salt.

The pharmaceutical compositions also may comprise suitable solid or gel phase carriers or excipients. Examples of such carriers or excipients include but are not limited to calcium

carbonate, calcium phosphate, various sugars, starches, cellulose derivatives, gelatin, and polymers such as polyethylene glycols.

Suitable liquid or solid pharmaceutical preparation forms are, for example, aqueous or saline solutions for inhalation, microencapsulated, encochleated, coated onto microscopic gold particles, contained in liposomes, nebulized, aerosols, pellets for implantation into the skin, or dried onto a sharp object to be scratched into the skin. The pharmaceutical compositions also include granules, powders, tablets, coated tablets, (micro)capsules, suppositories, syrups, emulsions, suspensions, creams, drops or preparations with protracted release of active compounds, in whose preparation excipients and additives and/or auxiliaries such as disintegrants, binders, coating agents, swelling agents, lubricants, flavorings, sweeteners or solubilizers are customarily used as described above. The pharmaceutical compositions are suitable for use in a variety of drug delivery systems. For a brief review of methods for drug delivery, see Langer, *Science* 249:1527-1533, 1990, which is incorporated herein by reference.

The CpG oligonucleotides and antigens may be administered per se (neat) or in the form of a pharmaceutically acceptable salt. When used in medicine the salts should be pharmaceutically acceptable, but non-pharmaceutically acceptable salts may conveniently be used to prepare pharmaceutically acceptable salts thereof. Such salts include, but are not limited to, those prepared from the following acids: hydrochloric, hydrobromic, sulphuric, nitric, phosphoric, maleic, acetic, salicylic, p-toluene sulphonc, tartaric, citric, methane sulphonc, formic, malonic, succinic, naphthalene-2-sulphonic, and benzene sulphonc. Also, such salts can be prepared as alkaline metal or alkaline earth salts, such as sodium, potassium or calcium salts of the carboxylic acid group.

Suitable buffering agents include: acetic acid and a salt (1-2% w/v); citric acid and a salt (1-3% w/v); boric acid and a salt (0.5-2.5% w/v); and phosphoric acid and a salt (0.8-2% w/v). Suitable preservatives include benzalkonium chloride (0.003-0.03% w/v); chlorobutanol (0.3-0.9% w/v); parabens (0.01-0.25% w/v) and thimerosal (0.004-0.02% w/v).

The pharmaceutical compositions of the invention contain an effective amount of a CpG oligonucleotide and optionally antigens and/or other therapeutic agents optionally included in a pharmaceutically-acceptable carrier. The term "pharmaceutically-acceptable carrier" means one or more compatible solid or liquid filler, dilutants or encapsulating substances which are suitable

for administration to a human or other vertebrate animal. The term "carrier" denotes an organic or inorganic ingredient, natural or synthetic, with which the active ingredient is combined to facilitate the application. The components of the pharmaceutical compositions also are capable of being commingled with the compounds of the present invention, and with each other, in a manner such that there is no interaction which would substantially impair the desired pharmaceutical efficiency.

The CpG oligonucleotides or antigens useful in the invention may be delivered in mixtures with additional adjuvant(s), other therapeutics, or antigen(s). A mixture may consist of several adjuvants in addition to the CpG oligonucleotide or several antigens or other therapeutics.

A variety of administration routes are available. The particular mode selected will depend, of course, upon the particular adjuvants or antigen selected, the particular condition being treated and the dosage required for therapeutic efficacy. The methods of this invention, generally speaking, may be practiced using any mode of administration that is medically acceptable, meaning any mode that produces effective levels of an immune response without causing clinically unacceptable adverse effects. Preferred modes of administration are discussed above.

The compositions may conveniently be presented in unit dosage form and may be prepared by any of the methods well known in the art of pharmacy. All methods include the step of bringing the compounds into association with a carrier which constitutes one or more accessory ingredients. In general, the compositions are prepared by uniformly and intimately bringing the compounds into association with a liquid carrier, a finely divided solid carrier, or both, and then, if necessary, shaping the product. Liquid dose units are vials or ampoules. Solid dose units are tablets, capsules and suppositories. For treatment of a patient, depending on activity of the compound, manner of administration, purpose of the immunization (i.e., prophylactic or therapeutic), nature and severity of the disorder, age and body weight of the patient, different doses may be necessary. The administration of a given dose can be carried out both by single administration in the form of an individual dose unit or else several smaller dose units. Multiple administration of doses at specific intervals of weeks or months apart is usual for boosting the antigen-specific responses.

Other delivery systems can include time-release, delayed release or sustained release delivery systems. Such systems can avoid repeated administrations of the compounds, increasing convenience to the subject and the physician. Many types of release delivery systems are available and known to those of ordinary skill in the art. They include polymer base systems such as poly(lactide-glycolide), copolyoxalates, polycaprolactones, polyesteramides, polyorthoesters, polyhydroxybutyric acid, and polyanhydrides. Microcapsules of the foregoing polymers containing drugs are described in, for example, U.S. Patent 5,075,109. Delivery systems also include non-polymer systems that are: lipids including sterols such as cholesterol, cholesterol esters and fatty acids or neutral fats such as mono-di-and tri-glycerides; hydrogel release systems; sylastic systems; peptide based systems; wax coatings; compressed tablets using conventional binders and excipients; partially fused implants; and the like. Specific examples include, but are not limited to: (a) erosional systems in which an agent of the invention is contained in a form within a matrix such as those described in U.S. Patent Nos. 4,452,775, 4,675,189, and 5,736,152, and (b) diffusional systems in which an active component permeates at a controlled rate from a polymer such as described in U.S. Patent Nos. 3,854,480, 5,133,974 and 5,407,686. In addition, pump-based hardware delivery systems can be used, some of which are adapted for implantation.

The present invention is further illustrated by the following Examples, which in no way should be construed as further limiting. The entire contents of all of the references (including literature references, issued patents, published patent applications, and co-pending patent applications) cited throughout this application are hereby expressly incorporated by reference.

Examples

Methods:

Proliferation assays. Briefly, spleen cells from BALB/c mice (4-18 weeks old) were cultured at 5×10^5 - 10^6 cells/ml in RPMI for 44 hours in 96-well microtiter plates, and then pulsed with 1 μ Ci of ^3H thymidine for 4 hours, before being harvested. The cpm was determined by scintillation counting. Proliferation assays were then performed to measure B cell activation essentially as previously described (Krieg, A.K., et al., 1995, *Nature*, 374:546).

Oligonucleotides. Non-stereoregular oligodeoxynucleotides were prepared by the standard phosphoroamidite method. Stereopure phosphorothioate oligodeoxynucleotides were prepared by the oxathiaphospholane method of Stec, et al. (Stec, W.J., et al., 1995, *J. Am. Chem. Soc.*, 117:12019). The sequence TCAACGTT (Seq ID No:21) was chosen for the study as a typical CpG motif with broad immune stimulatory effects representative of the broad family of CpG DNA.

Results. BALB/c spleen cells were cultured with stereopure oligonucleotides in which all of the internucleotide linkages are either R or S at the concentrations indicated in Table 2. Only the S stereoisomer (CG-S) induced B cell proliferation which was easily detectable at concentrations of just 1 $\mu\text{g/ml}$. In contrast, the R stereoisomer (CG-R) did not induce any proliferation even at concentrations of 24 $\mu\text{g/ml}$.

Table 2

Oligonucleotide	Concentration	cpm
CG-R	1 $\mu\text{g/ml}$	776 \pm 38
	6 $\mu\text{g/ml}$	603 \pm 16
	12 $\mu\text{g/ml}$	417 \pm 30
	24 $\mu\text{g/ml}$	405 \pm 74
CG-S	1 $\mu\text{g/ml}$	2744 \pm 938
	6 $\mu\text{g/ml}$	6070 \pm 2159
	12 $\mu\text{g/ml}$	11,529 \pm 2461
	24 $\mu\text{g/ml}$	34,394 \pm 9998
None		1258 \pm 273

A stereorandom octamer with the same sequence as that shown in Table 1 had reduced proliferative effect compared to the chirally pure S isomer. Furthermore, the addition of the R stereoisomer to cells that were being stimulated with the S stereoisomer caused a dose-dependent reduction in the proliferative effect. Thus, the R stereoisomer antagonizes the immune stimulation of the S isomer. Although Applicants do not wish to be bound by any mechanism, it is believed that one possible explanation for these results is that the binding protein which mediates the CpG effects binds to CpG DNA in a stereodependent fashion. Alternatively, the

transport mechanisms through which CpG DNA reach its site of action may be stereoselective. .
Regardless of the mechanism, Applicants have demonstrated that the S stereoisomer of CpG is involved in regulating the immune response.

The foregoing written specification is considered to be sufficient to enable one skilled in
5 the art to practice the invention. The present invention is not to be limited in scope by examples provided, since the examples are intended as a single illustration of one aspect of the invention and other functionally equivalent embodiments are within the scope of the invention. Various modifications of the invention in addition to those shown and described herein will become apparent to those skilled in the art from the foregoing description and fall within the scope of the
10 appended claims. The advantages and objects of the invention are not necessarily encompassed by each embodiment of the invention.

All references, patents and patent publications that are recited in this application are incorporated in their entirety herein by reference.

I claim:

an immunostimulatory nucleic acid having a sequence including at least the following formula:

wherein C and G are unmethylated, wherein X₁, X₂, X₃ and X₄ are nucleotides, wherein at least two nucleotides have a phosphate backbone modification forming a chiral center and wherein a plurality of the chiral centers have S chirality.

3. The composition of claim 1, wherein X₃X₄ are nucleotides selected from the group consisting of: TpT, TpA, TpG, ApA, ApG, GpA, and CpA.

5. The composition of claim 1, wherein the immunostimulatory nucleic acid is double stranded.

7. The composition of claim 1, wherein less than all of the chiral centers have S chirality.

9. The composition of claim 1, wherein at least 75% of the nucleotides have backbone

25 modifications.

10. The composition of claim 1, wherein at least 90% of the nucleotides have backbone modifications.

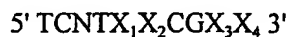
11. The composition of claim 1, wherein at least 60% of the chiral centers have S chirality.

5 12. The composition of claim 1, wherein at least 75% of the chiral centers have S chirality.

13. The composition of claim 1, wherein at least 90% of the chiral centers have S chirality.

14. The composition of claim 1, wherein the immunostimulatory nucleic acid is single
10 stranded.

15. The composition of claim 1, wherein the immunostimulatory nucleic acid has a sequence including at least the following formula:



wherein N is a nucleic acid sequence composed of from about 0-25 nucleotides.

15 16. The composition of claim 1, wherein the composition includes immunostimulatory nucleic acids having identical sequences.

17. The composition of claim 1, wherein the composition includes immunostimulatory nucleic acids having at least two different sequences.

18. The composition of claim 17, wherein the at least two sequences include a B-cell
20 activating sequence and an NK cell activating sequence.

19. The composition of claim 1, wherein the nucleic acid has less than or equal to 100 nucleotides.

20. The composition of claim 1, wherein the nucleic acid has between 8 and 40 nucleotides.

21. The composition of claim 1, further comprising an antigen.
22. The composition of claim 1, further comprising a cytokine.
23. The composition of claim 22, wherein the cytokine is selected from the group consisting of GM-CSF, IL-4, IL-18, IFN α , TNF α , Flt3 ligand, and IL-3.
- 5 24. The composition of claim 1, further comprising an antiviral.
25. The composition of claim 1, further comprising an antibacterial.
26. The composition of claim 1, further comprising a non-nucleic acid adjuvant.
27. The composition of claim 1, wherein the composition is formulated as a sustained release device.
- 10 28. The composition of claim 1, wherein the CpG formula is a palindrome.
29. A composition comprising:
- a double stranded immunostimulatory nucleic acid having a sequence on one strand including at least the following formula:
- $$5' X_1 X_2 CGX_3 X_4 3'$$
- 15 wherein C and G are unmethylated, wherein X_1 , X_2 , X_3 and X_4 are nucleotides, wherein at least two nucleotides have a phosphate backbone modification forming a chiral center and wherein a plurality of the chiral centers have S chirality.
30. The composition of claim 29, wherein $X_1 X_2$ are nucleotides selected from the group consisting of: GpT, GpG, GpA, ApA, ApT, ApG, CpT, CpA, CpG, TpA, TpT, and TpG.
- 20 31. The composition of claim 29, $X_3 X_4$ are nucleotides selected from the group consisting of: TpT, CpT, ApT, TpG, ApG, CpG, TpC, ApC, CpC, TpA, ApA, and CpA.
32. The composition of claim 29, wherein $X_1 X_2$ are nucleotides selected from the group consisting of: GpT, GpG, GpA and ApA; and $X_3 X_4$ are nucleotides selected from the group consisting of: TpT, CpT and ApT.

33. The composition of claim 29, wherein less than all of the nucleotides have a backbone modification.

34. The composition of claim 29, wherein less than all of the chiral centers have S chirality.

5 35. A method of inducing an antigen-specific immune response in a subject comprising:
administering to a subject an antigen and an immunostimulatory nucleic acid having a sequence including at least the following formula:



wherein C and G are unmethylated, wherein X_1 , X_2 , X_3 and X_4 are nucleotides, wherein at least
10 two nucleotides have a phosphate backbone modification forming a chiral center and wherein a plurality of the chiral centers have S chirality, in an amount effective to induce an antigen-specific immune response.

36. A method for redirecting a subject's immune response from a Th2 to a Th1 comprising:

15 administering to a subject an immunostimulatory nucleic acid having a sequence including at least the following formula:



wherein C and G are unmethylated, wherein X_1 , X_2 , X_3 and X_4 are nucleotides, wherein at least
20 two nucleotides have a phosphate backbone modification forming a chiral center and wherein a plurality of the chiral centers have S chirality, in an amount effective to redirecting the subject's immune response from a Th2 to a Th1.

37. A method for treating asthma in a subject, comprising:

administering to an asthmatic subject an effective amount for treating asthma in the
subject of an immunostimulatory nucleic acid having a sequence including at least the following
25 formula:



wherein C and G are unmethylated, wherein X_1 , X_2 , X_3 and X_4 are nucleotides, wherein at least two nucleotides have a phosphate backbone modification forming a chiral center and wherein a plurality of the chiral centers have S chirality.

- 5 38. A method for desensitizing a subject against the occurrence of an allergic reaction in response to contact with an allergen, comprising administering to a subject an immunostimulatory nucleic acid having a sequence including at least the following formula:



wherein C and G are unmethylated, wherein X_1 , X_2 , X_3 and X_4 are nucleotides, wherein at least
10 two nucleotides have a phosphate backbone modification forming a chiral center and wherein a plurality of the chiral centers have S chirality.

39. A method for activating an immune cell, comprising:

isolating an immune cell from a subject,

- contacting the immune cell with an effective amount to activate the immune cell of an
15 immunostimulatory nucleic acid having a sequence including at least the following formula:



wherein C and G are unmethylated, wherein X_1 , X_2 , X_3 and X_4 are nucleotides, wherein at least two nucleotides have a phosphate backbone modification forming a chiral center and wherein a plurality of the chiral centers have S chirality, and

- 20 readministering the activated immune cells to the subject.

40. The method of claim 39, wherein the immune cell is a lymphocyte.

41. The method of claim 40, further comprising contacting the immune cell with an antigen.

42. The method of claim 41, wherein the antigen is selected from the group consisting
25 of: a tumor antigen, a viral antigen, a bacterial antigen, and a parasitic antigen.

43. The method of claim 39, wherein the immune cell is a dendritic cell.

44. The method of claim 43, wherein the dendritic cell expresses a cancer antigen.

45. The method of claim 44, wherein the dendritic cell is exposed to the cancer antigen
5 *ex vivo*.

46. A method for activating a dendritic cell, comprising:

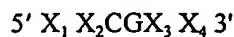
contacting a dendritic cell with an effective amount to activate a dendritic cell of an
immunostimulatory nucleic acid having a sequence including at least the following formula:



10 wherein C and G are unmethylated, wherein X_1 , X_2 , X_3 and X_4 are nucleotides, wherein at least
two nucleotides have a phosphate backbone modification forming a chiral center and wherein a
plurality of the chiral centers have S chirality.

47. A method for treating a cancer, comprising:

administering to a subject having a cancer an effective amount for treating the cancer of
15 an immunostimulatory nucleic acid having a sequence including at least the following formula:



wherein C and G are unmethylated, wherein X_1 , X_2 , X_3 and X_4 are nucleotides, wherein at least
two nucleotides have a phosphate backbone modification forming a chiral center and wherein a
plurality of the chiral centers have S chirality.

20 48. The method of claim 47, wherein the method is method for increasing the
responsiveness of a cancer cell to a cancer therapy and wherein the immunostimulatory nucleic
acid is administered in conjunction with an anti-cancer therapy.

49. The method of claim 48, wherein the anti-cancer therapy is an antibody.

50. A method for enhancing recovery of bone marrow in a cancer therapy subject, comprising:

administering to a subject undergoing or having undergone cancer therapy which damages the bone marrow an effective amount for enhancing the recovery of bone marrow of an immunostimulatory nucleic acid having a sequence including at least the following formula:



wherein C and G are unmethylated, wherein X_1 , X_2 , X_3 and X_4 are nucleotides, wherein at least two nucleotides have a phosphate backbone modification forming a chiral center and wherein a plurality of the chiral centers have S chirality.

51. In a method for stimulating an immune response in a subject having a cancer, the method of the type involving antigen dependent cellular cytotoxicity (ADCC), the improvement comprising:

administering to the subject an immunostimulatory nucleic acid having a sequence including at least the following formula:



wherein C and G are unmethylated, wherein X_1 , X_2 , X_3 and X_4 are nucleotides, wherein at least two nucleotides have a phosphate backbone modification forming a chiral center and wherein a plurality of the chiral centers have S chirality.

52. A method for inducing cytokine production in a subject comprising

administering to the subject an effective amount to induce a cytokine in the subject of an immunostimulatory nucleic acid having a sequence including at least the following formula:

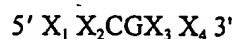


wherein C and G are unmethylated, wherein X_1 , X_2 , X_3 and X_4 are nucleotides, wherein at least two nucleotides have a phosphate backbone modification forming a chiral center and wherein a plurality of the chiral centers have S chirality.

53. The method of claim 52, wherein the cytokine is selected from the group consisting of Il-6, Il-12, IL18 TNF, IFN α and IFN- γ .

54. A method of stimulating natural killer cell lytic activity comprising

exposing a natural killer cell to an immunostimulatory nucleic acid to stimulate natural
5 killer cell lytic activity, the immunostimulatory nucleic acid having a sequence including at least
the following formula:



wherein C and G are unmethylated, wherein X_1 , X_2 , X_3 and X_4 are nucleotides, wherein at least
two nucleotides have a phosphate backbone modification forming a chiral center and wherein a
10 plurality of the chiral centers have S chirality.

55. A method of inducing a Th1-type immune response in a subject, comprising:

administering to the subject in order to induce a Th1 immune response a combination of
adjuvants, wherein the combination of adjuvants includes at least one immunostimulatory
nucleic acid having a sequence including at least the following formula:



wherein C and G are unmethylated, wherein X_1 , X_2 , X_3 and X_4 are nucleotides, wherein at least
two nucleotides have a phosphate backbone modification forming a chiral center and wherein a
plurality of the chiral centers have S chirality, and at least one non-nucleic acid adjuvant, and
wherein the combination of adjuvants is administered in an effective amount for inducing a Th1-
20 type immune response.

56. A method for inducing a mucosal immune response, comprising:

administering to a mucosal surface of a subject an effective amount for inducing a
mucosal immune response of an immunostimulatory nucleic acid having a sequence including at
least the following formula:



wherein C and G are unmethylated, wherein X_1 , X_2 , X_3 and X_4 are nucleotides, wherein at least two nucleotides have a phosphate backbone modification forming a chiral center and wherein a plurality of the chiral centers have S chirality, and

exposing the subject to an antigen to induce the mucosal immune response.

5 57. The method of claim 56, wherein the antigen is not encoded in a nucleic acid vector.

58. The method of claim 56, wherein the antigen is encoded by a nucleic acid vector.

59. The method of claim 56, wherein the mucosal surface is selected from the group consisting of an oral, nasal, rectal, vaginal, and ocular surface.

60. A composition comprising:

10 an immunoinhibitory nucleic acid having a sequence including at least the following formula:



15 wherein C and G are unmethylated, wherein X_1 , X_2 , X_3 and X_4 are nucleotides, wherein at least two nucleotides have a phosphate backbone modification forming a chiral center and wherein a plurality of the chiral centers have R chirality.

61. The composition of claim 60, wherein X_1X_2 are nucleotides selected from the group consisting of: TpA, ApA, ApC, ApG, and GpG.

62. The composition of claim 60, X_3X_4 are nucleotides selected from the group consisting of: TpT, CpT, TpA, TpG, ApA, ApG, GpA, and CpA.

20 63. The composition of claim 60, wherein X_1X_2 are nucleotides selected from the group consisting of: TpT, TpG, ApT, GpC, CpC, CpT, TpC, GpT and CpG; X_3 is a nucleotide selected from the group consisting of A and T and X_4 is a nucleotide, but wherein when X_1X_2 is TpC, GpT, or CpG, X_3X_4 is not TpC, ApT or ApC.

64. The composition of claim 60, wherein the immunoinhibitory nucleic acid is double stranded.

65. The composition of claim 60, wherein less than all of the nucleotides have a backbone modification.

5 66. The composition of claim 60, wherein less than all of the chiral centers have R chirality.

67. The composition of claim 60, wherein at least 50% of the nucleotides have backbone modifications.

68. The composition of claim 60, wherein at least 75% of the nucleotides have backbone
10 modifications.

69. The composition of claim 60, wherein at least 90% of the nucleotides have backbone modifications.

70. The composition of claim 60, wherein at least 60% of the chiral centers have R chirality.

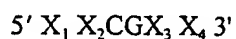
15 71. The composition of claim 60, wherein at least 75% of the chiral centers have R chirality.

72. The composition of claim 60, wherein at least 90% of the chiral centers have R chirality.

73. The composition of claim 60, wherein the immunoinhibitory nucleic acid is single
20 stranded.

74. A composition comprising:

a double stranded immunoinhibitory nucleic acid having a sequence on one strand including at least the following formula:



wherein C and G are unmethylated, wherein X_1 , X_2 , X_3 and X_4 are nucleotides, wherein at least two nucleotides have a phosphate backbone modification forming a chiral center and wherein a plurality of the chiral centers have R chirality.

75. The composition of claim 74, wherein X_1X_2 are nucleotides selected from the group consisting of: GpT, GpG, GpA, ApA, ApT, ApG, CpT, CpA, CpG, TpA, TpT, and TpG.

76. The composition of claim 74, X_3X_4 are nucleotides selected from the group consisting of: TpT, CpT, ApT, TpG, ApG, CpG, TpC, ApC, CpC, TpA, ApA, and CpA.

77. The composition of claim 74, wherein X_1X_2 are nucleotides selected from the group consisting of: GpT, GpG, GpA and ApA; and X_3X_4 are nucleotides selected from the group consisting of: TpT, CpT and ApT.

78. The composition of claim 74, wherein less than all of the nucleotides have a backbone modification.

79. The composition of claim 74, wherein less than all of the chiral centers have R chirality.

80. A method of preventing an immune response in a subject comprising:

administering to a subject having an excessive immune response an immunoinhibitory nucleic acid having a sequence including at least the following formula:



wherein C and G are unmethylated, wherein X_1 , X_2 , X_3 and X_4 are nucleotides, wherein at least two nucleotides have a phosphate backbone modification forming a chiral center and wherein a plurality of the chiral centers have R chirality, in an amount effective to prevent an immune response.

81. The method of claim 80, wherein the subject having an excessive immune response is a subject who has received an immune stimulating compound.

82. A method for treating a subject comprising:

administering to a subject having or at risk of having an inflammatory disease an immunoinhibitory nucleic acid having a sequence including at least the following formula:



5 wherein C and G are unmethylated, wherein X_1 , X_2 , X_3 and X_4 are nucleotides, wherein at least two nucleotides have a phosphate backbone modification forming a chiral center and wherein a plurality of the chiral centers have R chirality, in an amount effective to prevent induction of an immune response.

83. The method of claim 82, wherein the inflammatory disease is selected from the group
10 consisting of inflammatory bowel disease, autoimmune disease, gingivitis, psoriasis, and sepsis.

84. The method of claim 1, wherein the nucleic acid has a sequence including at least the formula GTCGTX₄.

15 85. A method for inducing antigen non-specific innate immune activation and broad spectrum resistance to infectious challenge.

SEQUENCE LISTING

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CpG ImmunoPharmaceuticals, Inc.

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and Related Methods

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INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 99/17100

A. CLASSIFICATION OF SUBJECT MATTER

IPC 7 C07H21/00 A61K31/70 C12N15/11

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 7 C07H A61K C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 96 39154 A (ISIS PHARMACEUTICALS INC ;COOK PHILLIP DAN (US); HOKE GLENN (US)) 12 December 1996 (1996-12-12) claims 1-6	1,3,5, 29,31, 60,62, 64,74,76
X	WO 96 19572 A (HYBRIDON INC ;TANG JINYAN (US); ROSKEY ALLYSEN M (US); AGRAWAL SUD) 27 June 1996 (1996-06-27) figure 1	60,64,74
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☐ Further documents are listed in the continuation of box C.

Patent family members are listed in annex.

* Special categories of cited documents:

A document defining the general state of the art which is not considered to be of particular relevance

E earlier document but published on or after the international filing date

L document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

O document referring to an oral disclosure, use, exhibition or other means

P document published prior to the international filing date but later than the priority date claimed

T later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

X document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

Y document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

S document member of the same patent family

Date of the actual completion of the international search

17 November 1999

Date of mailing of the international search report

23/11/1999

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Bardill, W

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

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